University of Naples "Federico II"

Department of Agriculture

Improvement and Management of Agro-forestry Resources

Soil Remediation

ENHANCING THE ECONOMIC LIABILITY OF BIOREFINERIES BY TRANSFORMING LIGNIN FROM LIGNOCELLULOSIC BIOMASSES INTO HUMIC-LIKE SUBSTANCES WITH PLANT PROMOTING BIOACTIVITY

Ph. D. THESIS

PRESENTED BY

Davide Savy

Tutor: Prof. Alessandro Piccolo

Coordinator: Prof. Guido D'Urso

2010-2013

Table of contents

SUMMARY

In this study, lignin from three biomasses (miscanthus, giant reed and a pre-treated giant reed) has been isolated by two different methodologies and thoroughly characterized to produce humic-like substances, which were shown to positively affect plant growth and physiology. The techniques employed concerned the use of either sulfuric acid (SAL) or hydrogen peroxide at alkaline pH (Ox). Low yields were obtained by the Ox-method (less than 30% of Klason lignin), whereas the amount of materials coming from the SAL-extractive method was even greater than the Klason lignin, maybe due to condensation reaction between lignin and plant cell wall carbohydrates. An aqueous solubility test at pH 7 showed that a large depolymerization was obtained with the Ox-method, due to oxidation of isolated lignin material. This was also supported by elemental analyses, which showed that Ox samples had large oxygen content. Conversely, the lignin materials obtained with the SAL method were less than 6% water-soluble, thereby indicating lower depolymerization fragmentation. Thermal analyses and both infrared and nuclear magnetic resonance (NMR) spectroscopies confirmed the effectiveness of the two methods in separating cellulose from lignin. Size exclusion chromatography conducted on the Ox lignins from the three substrates, revealed nominal molecular sizes lower than 3000 Da for all substrates. Gas-chromatography Mass Spectrometry (GC-MS) analyses conducted on Ox-lignins extracted from raw miscanthus and giant reed biomasses, revealed a predominant abundance of guaiacyl-type molecule, though the small presence of sugars was also noted. Finally, different concentrations of aqueous solutions of lignins isolated by the Ox method from miscanthus and giant reed raw biomass were tested on the early stage of maize seedlings growth, in order to verify whether thse humic-like fragments displayed a plant biostimulation. Germination was not effected by any concentration of both lignin substrates, while root and shoot length were significantly enhanced at specific concentrations. These results are

in accordance with some literature that indicated the bioativity of humic matter on plant growth parameters. These results suggest that water-soluble lignin fragments isolated from the lignocellulosic biomasses of this study exert hormone-like effects inasmuch as humic matter from soils of recycled biomasses. The employement of lignin residues from biorefineries in enhancing productivity of lignocellulosic biomasses in particular and agricultural crops in general appears to become an important contribution to the sustaiability of biorefineries.

Chapter 1

Introduction

The extraction of petroleum and its refining for the production of fuels (the so-called fossil fuels) and chemicals have raised serious environmental concerns, since it has contributed to climate changes (IPCC, 2007). The rise in energy demand due to ever larger world population and fossil fuels consumption has led to massive increase in greenhouse gases (GHG) emission in the atmosphere. Since GHG absorb infrared radiation from sunlight (Primack & Carotenuto, 2003), their increasing concentration produced an unnatural growth of global warming. The increase of greenhouse effect strongly influences agriculture, biocenosis, human health, climatic events, glaciers retreat, terrestrial and marine ecosystems, (EEA Report, 2004), and changes in species composition of certain habitats (Andrew & Hughes, 2004; D'Amen & Bombi, 2008). Moreover, since fossil fuels are a non-renewable resource, their progressive scarcity due to increase consumption has also economic implications. Hence, it is clear that a development system based on petrol consumption is not sustainable. For these reasons, reduction of dependence on petroleum-derived products and mitigation of global climate change are major challenges for researchers. Thus proposal of alternative energy supplies is of crucial importance. Among others, plant biomass is an attractive renewable resource as an alternative to fossil energy and it has received much attention from both scientists and politicians.

Plant biomass is mainly composed of lignocellulose, a mixture of cellulose, hemicellulose and lignin. These compounds are extracted and processed in biorefineries, which could be classified in first (FGB) and second generation biorefineries (SGB). In the former, the inputs for the production of fuels and chemicals are commonly either seeds and grains, such as maize and wheat, or oil-based crops, such as rapeseed (Cherubini, 2010). This has placed FGB in competition with

food and feed industries for the use of biomass and agricultural land, giving rise to serious ethical consequences. On the contrary, the entire plant of non-food crops is used to obtain fuels in the SGB. Furthermore, some Life Cycle Assessment studies indicated that development of SGB promises advantages over FGB-derived biofuels in terms of land-use efficiency and environmental performance (Searcy & Flynn, 2008; Fleming et al., 2006). This makes the SGB a suitable tool to obtain fuels and chemicals without losing sight of the sustainability concept. This hypothesis is also supported by the intuition according to which the net impact of the combustion of biofuels from SGB will be zero on greenhouse gas emission as long as forests are kept growing, because growing forests are able to sequester C through photosynthesis (Manomet, 2010). This concept is known as the carbon (C) neutrality of bioenergy.

This parameter is of crucial importance when choosing the plant to be grown for bioenergy purposes. Both miscanthus (*Miscanthus χ Giganteus*, Greef et Deuter) and giant reed (*Arundo donax*, L.) meet this requirement. They are both perennial rhizomatous herbaceous plants and show high productivity and favourable energy balance (Angelini et al., 2005; Heaton et al., 2004a). Furthermore, their cultivation may be more ecologically convenient than the growth of annual crops, since they need limited soil disturbance (sowing and tillage), thus reducing the risk of erosion (Heaton et al., 2004b) and improving soil carbon content and biodiversity. Moreover, perennial grasses can be conveniently grown without pesticide and fertilizer inputs, due to nutrient recycling when their rhizomes are left in soil (Lewandowski et al., 2000). All these reasons indicate that the appropriateness of these two plant cultivation in order to obtain lignocellulose to be worked up in biorefinery processing.

Lignocellulose can be considered as the largest source of hexose and pentose sugars in the biosphere for conversion in either bioethanol for fuel or various chemicals for industry (Holladay et al., 2007). To exploit the different lignocellulosic components, the cell wall constituents have to be

separated. Usually, once the cellulose is separated, the residue is burnt or discarded (Cherubini, 2010). This residue is mainly composed of hemicellulose and lignin. The latter constitutes about 15-30% by weight and the 40% by energy (Perlack et al., 2005) of the whole lignocellulosic biomass. Discarding lignin precludes this aromatic and precious photosyntate to be more profitably exploited. Thus, it is of crucial importance to find a new economic value to discarded lignin. Lignin has been proposed to be used as an input to yield either simple organic compounds, such as toluene, 4-hydroxy-3-methoxybenzaldehyde (vanillin) or phenol (Zakzeski et al., 2010), or biocomposites (Mansur et al., 2005).

Another possible way of using lignin is to employ its transformed products as plant growth promoters. It has been reported that lignin may function as plant stimulator (Ertani et al., 2001a, b; Popa et al., 2008), because it has some hormone-like effects, as both soil humic substances (HS) and humic-like substances (HULIS) have. It is widely recognized that HS, as well as HULIS positively affect plant growth and physiology in different ways. For instance, HS can increase NO_3^- uptake (Vaughan et al., 1985; Piccolo et al., 1992), due to their gibberellin (GA)-like activity (Nardi et al., 2000). Moreover, HS positively affect photosynthesis (Sladky et al., 1959; Ferretti et al., 1991; Merlo et al., 1991) and respiration in higher plants (Vaughan and Malcom, 1985). Piccolo et al. (1993), Canellas et al. (2008), and Busato et al. (2010) found that HS stimulated the elongation and proliferation of secondary roots in maize, while Dobbs et al., (2007) showed that HS influence the lateral root development in *Arabidopsis thaliana*, L. According to Canellas et al. (2002; 2009), these effects are due to an increase of the plasma membrane ATPase activity. Additionally, HS have beneficial effects on the growth of whole plants, even when experiments were conducted on a field- or on a greenhouse-scale, either used as a growth medium or applied in combination with a fertilizer. Verlinden et al. (2009) found that HS enhance plant uptake of nitrogen, phosphorous, calcium and magnesium and induce an increase of the overall crop production, when applied either as liquid solution to the soil or as a solid incorporated in mineral fertilizers. Tufencki et al. (2006)

reported a positive impact of HS soil application on the plant growth and nutrient contents of lettuce plants. Indeed, Atiyeh et al. (2002) tested the biological activity of HS when mixed either with mixed with a standard commercial potting medium or with vermiculite. They proved that HS greatly enhance the growth of tomato and cucumber seedlings, till a concentration of 500 mg kg^{-1}, then the development of plants was significantly decreased. Also the foliar application of HS can significantly promote the growth of root and shoot, as shown by Silva-Matos et al. (2012). They have also reported an increase in the leaf chlorophyll content of net-house grown watermelon seedlings. Finally, HS are also able to induce plant resistance to either biotic or abiotic stresses. Interestingly, HS biotic and abiotic stresses. Kesba & El-Betagi (2012) reported a high resistance of grape toward nematode infections, while Loffredo et al. (2008) showed a positive effect of HS in inhibiting the growth of *Sclerotinia sclerotiorum* mycelium and contemporarily an increase of the growth of one of its antagonists, *Trichoderma viride*. García et al. (2012) showed that HS increased the tolerance of rice toward water stress.

In order to be employed in fine chemical transformations and bioactive processes, lignin has to be firstly separated from the rest of lignocellulose and extensively characterized. One of the extraction methods employed is based on the use of acidic pH solutions. The most employed inorganic acids are sulphuric (H_2SO_4) and hydrochloric (HCl) acids (Sun & Cheng, 2002). These methods are commonly used to yield fermentable sugars. It was found that this kind of treatment enhances the enzymatic digestibility of lignocellulosic biomass. It is, however, important to remind that the acid hydrolysis can produce furfural, which is a well-known inhibitor of the microbial growth (Kumar et al, 2009). Even if this method employs hazardous chemicals and is quite expensive, it is still of interest (Matsushita et al., 2011) and it has also been patented (Clausen & Gaddy, 1993).

Another method to isolate high-purity lignin is based on the use of hydrogen peroxide solutions at alkaline pH. This method was developed in the paper-making industry as an alternative to the chlorine and chlorine-based reagents, which present many environmental concerns due to the formation of organochlorinated compounds (Sjöström, 1993). The use of strong bases, such as NaOH or $Ca(OH)_2$, instead, is more environmentally-friendly. Also, this treatment provides effective delignification and chemical swelling of fibrous cellulose. Studies on the treatment of straw with alkaline reagents, particularly NaOH showed that up to 50% of *Poaceae* lignin was readily solubilized by 1.5% aqueous NaOH solution at room temperature (Beckman et al., 1923).

The addition of peroxide to the reaction mixture significantly enhances lignin hydrolysis due to cell walls disruption and solubilisation of hemicellulose and lignin (Yamashita et al., 2010). Moreover, α-aryl ether linkages in phenolic units are readily cleaved by the conversion of phenolate units into the corresponding quinone methide intermediates (Figure 1). Fragmentation leads to the generation of phenolic hydroxyl groups and catechol, and, in turn, the lignin fragments are rendered soluble in alkaline water solution (Chakar et al., 2004). Lignin is then easily recovered by lowering the pH or by concentration, filtration, and drying (Holladay et al., 2007). Generally, the oxidative alkaline method increases degradability of cell walls due to cleavage of either lignin-hemicellulose or lignin-cellulose bonds (Yamashita et al., 2010).

The bleaching action of hydrogen peroxide at pH higher than 7 is mainly attributed to hydroperoxide ion (HOO^-), which dominates under alkaline conditions (pK_a=11.6) and is responsible for the elimination of chromophore groups from lignin. It is assumed that under alkaline conditions, the reaction between hydrogen peroxide and lignocellulose yields low molecular weight, water-soluble oxidation phenols and hemicelluloses, which are also solubilised. The lignin resulting from this process seems to be lower in molecular weight and richer in carbonyl and carboxyl functionalities than Kraft or sulfite lignin (Holladay et al., 2007).

The objective of this work was to compare the physical-chemical characteristics of the lignin extracted by an oxidative alkaline procedure with those extracted with sulfuric acid from three biomasses: miscanthus, giant reed and a microbially pre-treated giant reed. The lignins isolated were characterized by Attenuated Total Reflectance Infrared Fourier Transform spectroscopy, solid state ^{13}C-Nuclear Magnetic Resonance spectroscopy, thermal analysis and high performance size exclusion chromatography. Moreover, their solubility in water was quantitatively assessed. Molecular studies on lignin isolated from the two different biomasses were conducted by first breaking intramolecular lignin linkages and then characterizing the fragments by gas chromatography-mass spectrometry and by ^{31}P-Nuclear Magnetic Resonance spectroscopy. The lignins isolated from two raw biomasses by the alkaline oxidative process were also used in biological assays to determine their effect on the germination of maize seeds. To this purpose, primary and lateral roots elongation as well as shoot development in maize seedlings have also been measured.

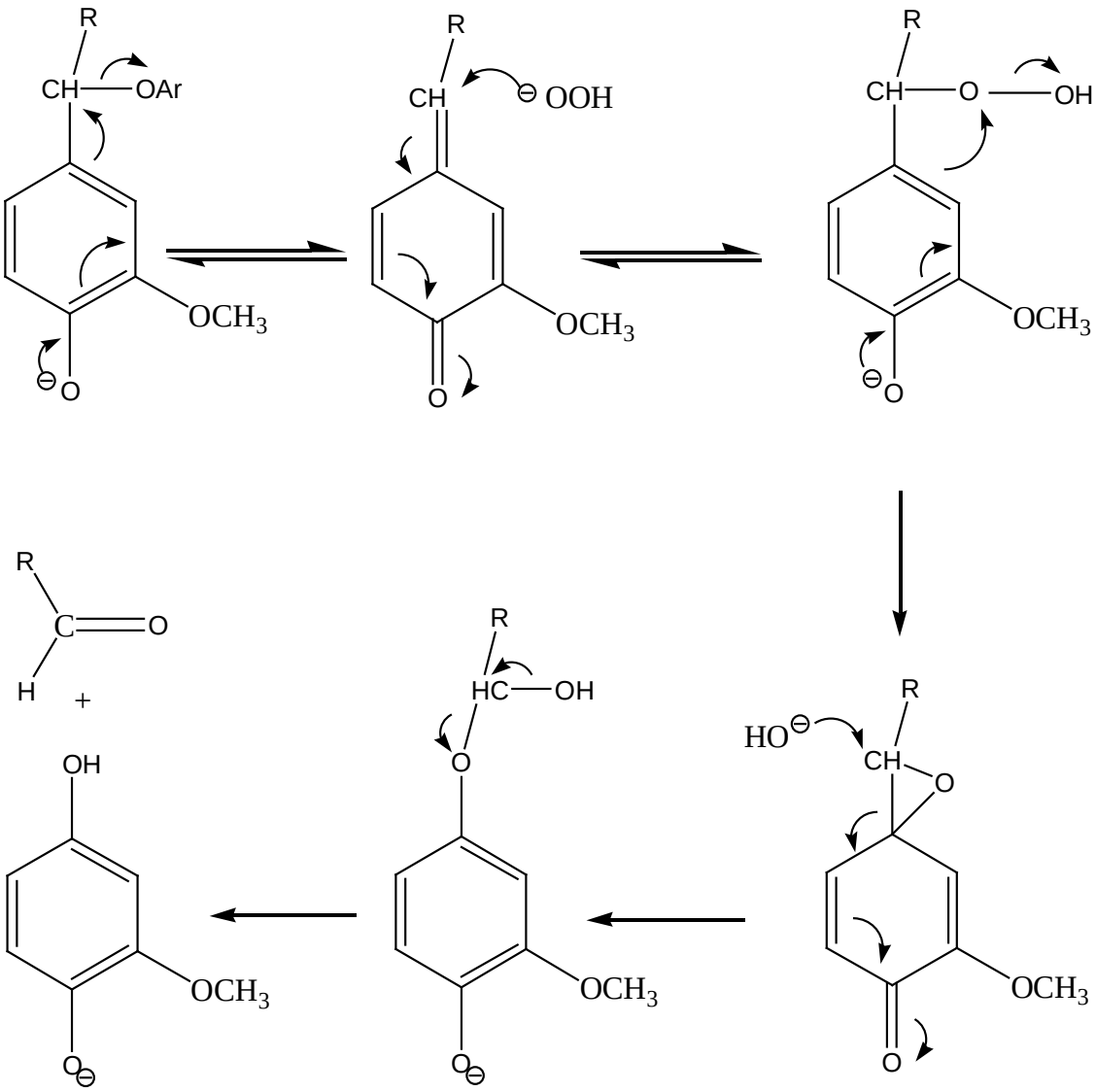


Figure 1. Cleavage of α-aryl ether linkage of a guaiacyl-type lignin dimer based on Dakin-like mechanism in alkaline solution.

References

Andrew, N R & Hughes, L (2004) Species diversity and structure of phytophagous beetle assemblages along a latitudinal gradient: predicting the potential impacts of climate change. Ecological Entomology, 29, 527–542.

Angelini, L G; Ceccarini, L; Bonsri, E (2005) Biomass yield and energy balance of giant reed (*Arundo donax*, L.) cropped in central Italy as related to different management practices Europ. J. Agronomy 22, 375–389.

Atiyeh, R M; Lee, S; Edwards, C A; Arancon, N Q; Metzger, J D (2002) The influence of humic acids derived from earthworm-processed organic wastes on plant growth. Bioresource Technology, 84, 7-14.

Beckmann, E, Liesche, O; Lehmann, F (1923) Qualitative und quantitative Unterschiede der Lignin einiger Holz- und Stroharten. Biochemische Zeitschrift, 139, 491–508.

Busato, J G; Zandonadi, D B; Dobbss, L B; Façanha, A R; Canellas, L P (2010) Humic substances isolated from residues of sugar cane industry as root growth promoter. Scientia Agricola, 67, 206-212.

Canellas, L P; Olivares, F L; Okorokova- Façanha, A L; Façanha, A R (2002) Humic Acids Isolated from Earthworm Compost Enhance Root Elongation, Lateral Root Emergence, and Plasma Membrane H+-ATPase Activity in Maize Roots. Plant Physiology, 13, 1951-1957.

Canellas, L P; Spaccini, R; Piccolo, A; Dobbss, L B; Okorokova- Façanha, A L; Santos, G A; Olivares, F L; Façanha, A R (2009) Relationships between chemical characteristics and root growth promotion of humic acids isolated from bazilian oxisols. Soil Science, 174, 611-620.

Canellas, L P; Zandonadi, D B; Busato, J G; Baldotto, M A; Simoes, M L; Martin-Neto, L; Façanha, A R; Spaccini, R; Piccolo, A (2008) Bioactiviy and Chemical Characteristics of Humic Acids from Tropical Soils Sequence. Soil Science, 179, 624-637.

Chakar, F S, Ragauskas, A J (2004). Review of current and future softwood kraft lignin process chemistry. Industrial Crops and Products. 20, 131–141.

Cherubini, F (2010) The biorefinery concept: Using biomass instead of oil for producing energy and chemicals. Energy Conversion and Management 51, 1412–1421.

Clausen, E C; Gaddy, J L (1993) Concentrated sulfuric acid processing for converting lignocellulosic materials to sugars, U. S. Pat. 5,188,673.

D'Amen, M & Bombi, P (2009) Global warming and biodiversity: Evidence of climate-linked amphibian declines in Italy. Biological Conservation, 142, 3060–3067.

Dobbss, L B; Medici, L O; Peres, L E P; Pino-Nunes, L E; Rumjanek, V M; Façanha, A R; Canellas, L P (2007) Changes in root development of Arabidopsis promoted by organic matter from oxisols. Annals of Applied Biology, 151, 199-211.

EEA – European Environment Agency (2004) Impacts of Europe's changing climate. EEA Report 2/2004, Copenhagen.

Ertani, A; Francioso, O; Tugnoli, V; Righi, V; Nardi, S (2011a) Effect of Commercial Lignosulfonate-Humate on *Zea mays* L. Metabolism. Journal of Agricultural and Food Chemistry, 59, 11940 – 11948.

Ertani, A; Schiavon, M; Altissimo, A; Franceschi, C; Nardi, S (2011b) Phenol-containing organic substances stimulate phenylpropanoid metabolism in Zea mays. Journal of Plant Nutrition and Soil Science, 174, 496-503.

Ferretti, M; Ghisi, R; Nardi, S; Passera, C (1991) Effect of humic substances on photosynthetic sulphate assimilation in maize seedlings. Canadian Journal of Soil Science, 71, 239–242.

Fleming, J S; Habibi, S; MacLean, H L (2006) Investigating the sustainability of lignocellulose-derived fuels for light-duty vehicles. Transportation Research Part D: Transport and Environment, 11 (146–159).

García, A C; Santos, L A; Izquierdo, F G; Sperandio, M V; Castro, R N; Berbara, R L L (2012) Vermicompost humic acids as an ecological pathway to protect rice plant against oxidative stress. Ecological Engineering, 47, 203-208.

Heaton E, Voigt T, Long S P (2004b) A quantitative review comparing the yields of two candidate C4 perennial biomass crops in relation to nitrogen, temperature and water. Biomass and Bioenergy, 27, 21–30.

Heaton, E A;Clifton-Brown, J;Voigt, T; Jones M B; Long S P (2004a) Miscanthus for renewable energy generation: European Union experience and projections for Illinois. Mitigation Adapt. Strategies Global Change, 9, 433–451.

Holladay, J E; Bozell, J J; White, J F; Johnson, D (2007). Top Value-Added Chemicals from Biomass Volume II—Results of Screening for Potential Candidates from Biorefinery Lignin. Pacific Northwest National Laboratory, U. S. Dempartment of Energy.

IPCC (2007) Climate Change 2007: Synthesis Report. IPCC, Geneva, Switzerland.

Kesba, H H & El-Betagi, H S (2012) Biochemical changes in grape rootstocks resulted from humic acid treatments in relation to nematode infection. Asian Pacific Journal of Tropical Biomedicie, 2, 287-293.

Kumar, P; Barrett, D M; Delwiche' M J; Stroeve, P (2009) Methods for Pretreatment of Lignocellulosic Biomass for Efficient Hydrolysis and Biofuel ProductionIndustrial & Engineering Chemistry Research 48, 3713–3729.

Lewandowski, I; Clifton-Brown, J C; Scurlock, J M O; Huisman W (2000) Miscanthus: European experience with a novel energy crop. Biomass and Bioenergy, 19, 209–27.

Loffredo, E; Berloco, M; Senesi, N (2008) The role of humic fractions from soil and compost in controlling the growth in vitro of phytopathogenic and antagonistic soil-borne fungi. Ecotoxicology and Environmental Safety, 69, 350–357.

Manomet, T (2010) Biomass Sustainability and Carbon Policy Study. In: Massachusetts Biomass Sustainability and Carbon Policy Study: Report to the Commonwealth of Massachusetts Department of Energy Resources (ed. Walker T), Contributors: Cardellichio, P; Colnes, A; Gunn, J; Kittler, B; Perschel, R; Recchia, C; Saah, D, and Walker, T. Manomet Center for Conservation Sciences, Brunswick, ME.

Mansur, H S; Mansur, A A P; Bicallho, S M C M (2005) Key Engineering Materials, 284-286, 745-748.

Matsushita, Y; Inomata, T; Takagi, Y; Hasegawa, T;Fukushima, K (2011) Conversion of sulfuric acid lignin generated during bioethanol production from lignocellulosic materials into polyesters with ε–caprolactone. Journal of Wood Science, 57, 214-218.

Merlo, L; Ghisi, R; Rascio, N; Passera, C (1991) Effects of humic substances on carbohydrate metabolism of maize leaves. Canadian Journal of Plant Science, 71, 419–425.

Nardi, S; Pizzeghello, D; Gessa, C; Ferrarese, L; Trainotti, L; Casadoro, G (2000) A low molecular weight humic fraction on nitrate uptake and protein synthesis in maize seedlings. Soil Bology & Biochemistry, 32, 415–419.

Perlack, R D; Wright, L L; Turhollow, A F; Graham, R L; Stokes, B J; Erbach, D C U S. Department of Energy, Biomass as Feedstock for a bioenergy and bioproducts industry: the technical feasibility of a billion-ton annual supply, 2005.

Piccolo A., S. Nardi, G. Concheri. Structural characteristics of humic substances as related to nitrate uptake and growth regulation in plant systems. Soil Biology and Biochemistry, 24:373-380 (1992).

Piccolo A., G. Celano, G. Pietramellara. Effects of fractions of coal derived humic substances on seed germination and seedling growth. Biology and Fertility of Soils, 16:11-15, (1993).

Primack, R B & Carotenuto, L. In: Conservasione della Natura. Zanichelli Publications, Bologna. Italy, 2003.

Popa, V I; Dumitru, M; Volfa, I; Anghel, N (2008) Lignin and polyphenols as allelochemicals. Industrial crops and products, 27, 144–149.

Searcy, E; Flynn, P C (2008) Processing of Straw/Corn Stover: comparison of life cycle emissions. International Journal of Green Energy, 5 (423–437).

Silva-Matos, R R S; Cavalcante, I H L; Jùnior, G B S; Albano, F G; Cunha, M S; Beckman-Cavalcante, M Z (2012) Foliar Spray of Humic Substances on Seedling Production of Watermelon cv. Crimson Sweet. Journal of Agronomy, 11, 60-64.

Sjöström, E (1993). *Wood Chemistry: Fundamentals and Applications*. Academic Press. ISBN 012647480X. OCLC 58509724.

Sjöström, E (1993). *Wood Chemistry: Fundamentals and Applications*. Academic Press. ISBN 012647480X. OCLC 58509724.

Sladky, Z (1959) The effect of extracted humus substances on growth of tomato plants. Biologia Plantarum, 1, 142–150.

Sun, Y; Cheng, J (2002) Hydrolysis of lignocellulosic materials for ethanol production: a review. Bioresource Technology 83 1–11.

Tufencki, S; Turkmen, O; Sonmez, F; Erdinc, C; Sensoy, S (2006) Effects of humic acid doses and application times on the plant growth, nutrient and heavy metal contents of lettuce grown on sewage sludge-applied soils. Fresenius Environmental Bulletin 15, 295–300.

Vaughan, D & Malcom, R E (1985) Influence of humic substances on biochemical processes in plants. In: Vaughan, D & Malcom, R E (Eds.), Soil Organic Matter and Biological Activity, Martinus Nijhoff/Junk W, Dordrecht, The Netherlands, pp. 77–108.

Vaughan, D & Malcom, R E (1985) Influence of humic substances on growth and physiological processes. In: Vaughan, D & Malcom, R E (Eds.), Soil Organic Matter and Biological Activity, Martinus Nijhoff/Junk W, Dordrecht, The Netherlands, pp. 37–76.

Verlinden, G; Pycke, B; Mertens, J; Debersaques, F; Verheyen, K; Baert, G; Bries, J; Haesaert, G (2009) Application of Humic Substances Results in Consistent Increases in Crop Yield and Nutrient Uptake. Journal of Plant Nutrition, 32, 1407-1426.

Yamashita, Y, Shono, M, Sasaki, C, Nakamura, Y (2010). Alkaline peroxide pretreatment for efficient enzymatic saccharification of bamboo Carbohydrate Polymers 79, 914–920.

Zakzeski, J; Bruijnincx, P C A; Jongerius, A L; Weckhuysen, B M (2010) The Catalytic Valorization of Lignin for the Production of Renewable Chemicals. Chemical Reviews, 110, 3552–3599.

Chapter 2

Experimental section

Biomasses. Giant reed (*A. donax*, L) sample (AD) originated from the University of Naples "Federico II" experimental farm at Bellizzi (Salerno, Italy), whereas sample of *Miscanthus* χ *Giganteus* (MG) was provided by Phytatec Ltd (Birmingham, UK). A particular lignocellulosic material (RL) already undergone a microbial treatment for cellulose release was provided by the Mossi and Ghisolfi Group of Tortona (Alessandria, Italy).

Elemental analysis. The elemental composition (C, N, H) of the lignin obtained from oxidative hydrolysis was carried out using an elemental analyzer EA 1108 Elemental Analyzer (Fisons' Instruments).

Sulfuric acid hydrolysis. Lignocellulosic samples were treated with H_2SO_4 to remove cellulose as proposed by Cheshire & Mundie (1966). Briefly, 1 g was treated with 30 mL of 12 M H_2SO_4 solution for 16 hours at room temperature and under stirring. Then, water was added to the suspension to lower the H_2SO_4 concentration down to 1 M and kept continuously stirred for 3 hours at 105°C. The resulting lignin residue was centrifuged at 10,000 rpm for 30 min, dialyzed against deionized water, freeze-dried and stored in dried conditions before further analysis. The lignins obtained from RL, AD and MG with such H_2SO_4 treatment will be referred to as SAL-RL, SAL-AD and SAL-MG, respectively.

Alkaline oxidative hydrolysis. The alkaline oxidative lignin extraction has been carried out as previously reported by Sun et al. (2000). 5.0 g of every biomass sample was placed in a 150 mL of distilled water containing a 2 % H_2O_2 (v/v) solution. Then, the mixture pH was raised to 11.5 with a 4 M NaOH solution and stirred at 50°C. After 24 hours, the reaction mixture was centrifuged

(10,000 rpm x 10 min). The pH of the supernatant (lignin and hemicellulose) was lowered to 5.5, while 3 volumes of ethanol were added to flocculate the hemicellulose. The mixture was then filtered through a cellulose filter (Carlo Erba). The ethanol in the filtrate was removed by rotoevaporation, and the pH lowered to 1.5-2.0 with 6 N HCl to precipitate the lignin. The lignin was then freeze-dried and stored in dried conditions for further analyses. The lignins obtained from RL, AD and MG were referred to as Ox-RL, Ox-AD and Ox-MG, respectively.

Klason lignin determination. Lignin content in raw biomasses was determined by using the Klason assay as by the ASTM Standard method E 1721 (ASTM, 1998). Briefly, 300 mg of each sample were treated with 3.0 mL of 72% H_2SO_4 at 30°C for 2 h. The hydrolysed material is then transferred to a glass bottle and the acid was diluted until 4% concentration. Bottles were autoclaved in these sealed bottles for 1 h at 121°C. After completion of autoclave cycle, samples were filtered through a filtering crucible and dried at 105°C for 2 h until constant weight is reached. Finally, the crucibles containing the acid-insoluble lignin and the acid-insoluble ash are placed in a muffle furnace and ignited at 575±25°C for at least 3 h. Heating rate of 10°/min has been used to prevent flaming. The weight of crucibles with only acid-insoluble ash is recorded to the nearest 0.1 mg. Klason lignin content was determined as follows

$$\%\,\text{lignin} = \frac{W2 - W3}{W1 \times \frac{T_{105}}{100}} \times 100 \qquad (1)$$

where

W_1: initial sample weitght

W_2: weight of crucible + acid-insoluble lignin + acid-insoluble ash

W_3: weight of crucible + acid-insoluble ash

$T_{105}/100$: % of total solids determined at 105°C.

All analyses were conducted in duplicate

Solubility assay. The solubility of lignins extracted was evaluated by placing 0.1 g of lignin in 100 mL of distilled water and titrating the solution to pH 7 with 0.1 N NaOH. The solid residue remaining after titration was separated by centrifugation (10,000 rpm x 10 min), died in oven (40°C) and weighted. The amount of soluble material was obtained by subtracting the weight of the residual pellet from that of initial lignin.

High performance size exclusion chromatography (HPSEC). The HPSEC system was composed by a Shimadzu LC-10-AD pump equipped with a Rheodyne rotary injector and 100-μL sample loop and two detectors in series: a UV/VIS detector (LC295, Perkin-Elmer, wavelength set at 280 nm) and a Refractive Index (RI) detector (Refractomonitor IV, Fisons Instrument). The chromatographic column was a Biosep SEC s2000 300 x 7.80 mm (Phenomenex, USA), preceeded by a Biosep S-2000 35 x 7.80 pre-column (Phenomenex, USA) and a 2 mm inlet filter. The elution flow rate was set to 0.6 mL min^{-1} for an eluting solution made of 0.1 M NaH_2PO_4 solution, buffered at pH 6.5 with 0.1 M NaOH, The eluent was added with NaN_3 up to 0.3 g L^{-1} and filtered through 0.45 μm Millipore filter. A Unipoint Gilson Software was used to record each chromatographic run. The column calibration was obtained with the following sodium polystyrene sulfonates of known molecular masses: 123,000, 16,900, 6,780 and 1,200 Da. Ferulic acid (194 Da) and catechol (110 Da) have been also used as standards for the lowest molecular weights. The calibration curves provided the following relations between molecular weight (MW) and column retention time (RT): log MW = -0.14 * RT + 6.339, for the UV detector, and, log MW = -0.138 * RT + 6.338, for the RI detector. The coefficient of determination (R^2) for both curves was 0.97. Weight-average molecular weight (M_w), number-average molecular weight (M_n), and polydispersity ($P = M_w/ M_n$) were calculated using as described elsewhere (Yau et al., 1979).

Thermal analysis. Thermogravimetric analysis (TGA) and differential scanning calorimetry (DSC) curves were obtained by air combustion of sample (5-10 mg) in a simultaneous thermal analyzer (STA 6000-Perkin Elmer). The initial and final temperatures were 30 and 700°C, respectively, with an increasing rate of 10°C/min.

Attenuated Total Reflectance Infrared Fourier Transform spectroscopy (ATR-IR). The ATR-IR spectra were recorded with a Perkin Elmer 1720-X FT-IR spectrometer, equipped with a Perkin-Elmer Attenuated Total Reflectance accessory, accumulating up to 8 scans with a resolution of 4 cm^{-1}.

^{31}P-liquid- and ^{13}C-solid-state Nuclear Magnetic Resonance (NMR) spectroscopy. Samples (12.0 mg) were placed in NMR tubes with 674 μL of $CDCl_3$,13 μL of N,N-dimethylformamide and 13 μL f pyridine. Triphenyl phosphate (TPP, 400 μL in $CDCl_3$, 5 g/L, Sigma-Aldrich) and chromium (III) acetylacetonate (200 μL in $CDCl_3$, 1.6 g/L, Sigma-Aldrich) were used to calibrate the axis and as relaxing reagent, respectively. Finally, 100 μL of 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxa-phospholane (Sigma-Aldrich) were added to the solution. A 400 MHz Bruker Avance spectrometer, equipped with a 5 mm Bruker Inverse Broad Band (BBI) probe, working at ^{1}H frequency of 400.13 MHz, was employed to conduct ^{31}P liquid-state NMR measurements at a temperature of 298±1 K. ^{31}P-NMR spectra were acquired by adopting an inverse gated pulse sequence, including 80 μs length (15.6 dB power level) Waltz16 scheme in order to decouple phosphorous from proton nuclei. The experiments were acquired by selecting an initial 45° pulse length of 7.8 μs and a spectral width of 400 ppm (64,935.066 Hz), where the on-resonance frequency was set to 100 ppm. Each spectrum consisted in a 10 s thermal delay, 304 transients, 8 dummy scans and 65,536 time domain points. All spectra were baseline corrected and processed by both Bruker Topspin Software (v.2.1) and MestReC NMR Processing (v. 4.9.9.9). The Fourier Transform of FIDs (Free Induction Decays) was conducted by applying a 1.5 Hz

exponential multiplication as well as no zero-filling execution. ^{31}P frequency axis was calibrated by associating the value of -17.495 ppm to the TPP resonance.

The ^{13}C-NMR spectra of the different lignins were recorded by Cross Polarization Magic Angle Spinning (CPMAS) solid-state spectroscopy with a Bruker AVANCE 300 using a rotating speed of 13±1 kHz, a recycle time of 2 s, an acquisition time of 33 ms and 4,000 scans and a contact time of 1 ms. A ^{1}H ramp sequence was used to account for possible inhomogeneity of the Hartmann-Hahn condition. The free induction decays (FID) were transformed by applying a 4k zero filling and an exponential filter function with a line broadening of 100 Hz. Spectra processing was conducted by the 4.9.9.9 Mestre-C software.

Proton spin–lattice relaxation times ($T1\rho(H)$) were measured and calculated by using the following equation:

$$I_{(t)} = I_0 \exp[-t_{SL}/ T1\rho(H)] \qquad (2)$$

where t_{SL} is the variable spin lock time, $I_{(t)}$ is the signal area at t^{th} spin lock time, and I_0 is the signal area in absence of any relaxation. The t_{SL} values were comprised between 10 and 10,000 ms.

Derivatization followed by reductive cleavage (DFCR). To study the lignins at a molecular level, the DFRC procedure has been implemented. The analytical method was almost identical to that reported by Lu & Ralph (1997). 50 mg of lignin were treated with 25 mL of a 1:9 (v/v) acetyl bromide solution for 3 h at 50°C under magnetic stirring. After this step, the solvent was evaporated under vacuum. Then, the sample was dissolved in a 5:4:1 dioxane:acetic acid:water solution and Zn powder (250 mg) was added to this solution. This step allows the β-O-4 aryl alkyl ether linkages to be broken down and double bond to be formed between Cα and Cβ. After 30 min at room temperature, the mixture was quantitatively transferred to a saturated NH_4Cl solution (7.5 mL) in a separating funnel using HPLC-grade dichloromethane (7.5 mL). The aqueous layer was extracted

with dichloromethane (2 x 7.5 mL). The two extracts were dried with anhydrous Na_2SO_4 and then the solvent was evaporated to dryness under vacuum. The solution was then transferred to a 25 mL volumetric flask with dichloromethane and 5 mL of this solution were subjected to acetylation. Acetylation was carried out as follows: the 5 mL dichloromethane solution was dried under N_2 stream and, then, pyridine (0.5 mL) and acetyl acetate (0.3 mL) were added. The reaction lasted 30 min at room temperature. After that, the mixture was dried under N_2 stream. Finally, 10 μL of a 77.3 ppm hexadecane solution (in dichloromethane) and 990 μL of dichloromethane were added for GC-MS analysis.

Gas chromatography-Mass spectrometry. An aliquot of the previously prepared solution (2 μL) was injected (splitless mode) into a Perkin-Elmer Autosystem XL gas chromatograph equipped with a RTX-5MS WCOT capillary column, (Restek, 5% diphenyl-95% dimethylpolysiloxane, length 30 m, 0.25 mm ID, and 0.25 μm df) that was coupled, through a heated transfer line (250 °C), to a PE Turbomass-Gold quadrupole mass spectrometer. The injection port temperature was 270 °C, and the oven temperature was raised from 100 to 280 °C with a gradient of 8 °C/min. The internal standard used was hexadecane and the estimated correction factor was 6.96.

Bioactivity assay. The biological activity of lignin was evaluated by treating maize (*Zea mays*, L.) seeds with 0 (control), 0.1, 1, 10 and 100 ppm of C. After 96 h, germination percentage was evaluated by considering as germinated the seeds that showed an opened testa and a radicle long at least 1 mm (Morrison et al., 1998). Then, seedlings were scanned with an Epson Perfection V700 modified flat-bed scanner and primary root, lateral root and shoot length were measured with WinRhizo version 2012b (Regent Instruments, Inc.).

Statistical analysis. The comparison of medians of solubility (four replicates per each treatment) was carried out by performing the Kruskal-Wallis one-way analysis of variance statistical test (Kruskal & Wallis, 1952). This test was accomplished by using Microsoft Excel v. 2010 (Microsoft Corporation, WA). Analysis of variance with One-Way ANOVA and Tukey's

range test were performed to compare means among the treatments in the bioactivity assays. These tests were carried out by using Statistix version 9 (Analytical Software, Tallahassee, FL). Both the hypothesis tests were executed at the p-value less than or equal to 5%.

References

ASTM (1998), in Annual Book of ASTM Standards, ASTM Method E 1721-95, vol. 11.05, American Society for Testing and Materials, West Conshohocken, PA.

Cheshire, M. V. & Mundie, M. (1966). "The hydrolytic extraction of carbohydrates from soil by sulphuric acid". Journal of Soil Science, 17, 372-3814.

Kruskal, W H; Wallis, W A (1952) Use of Ranks in One-Criterion Variance Analysis. Journal of the American Statistical Association, 47, 583-621.

Lu, F; Ralph, J (1997) Derivatization Followed by Reductive Cleavage (DFRC method), a New Method for Lignin Analysis: Protocol for Analysis of DFRC monomers. Journal of Agricultural and Food Chemistry, 45, 2590 - 2592.

Morrison, D A; McClay, C; Porter, C; Rish, S (1998) The Role of the Lens in Controlling Heat-induced Breakdown of Testa-imposed Dormancy in Native Australian Legumes. Annals of Botany 82, 35–40.

Sun, R, Tomkinson, J, Wang, S; Zhu, W (2000). Characterization of lignins from wheat straw by alkaline peroxide treatment. Polymer Degradation and Stability 67, 101-109.

Yau W W, Kirkland J J, Bly D D, eds. Modern size exclusion chromatography. Wiley, New York, 1992.

Chapter 3

Solubilization and characterization of lignin separated from lignocellulose-rich plant biomasses

Introduction

Plant biomass is a natural renewable resource that can be converted into useful materials and energy. Lignocelluloses can be considered as the largest sources of hexose and pentose sugars for potential conversion in either bioethanol for fuels or various chemicals for industry (Holladay et al., 2007). Bioethanol has been massively obtained from food crops such as wheat and maize (Kaparaju et al., 2008; Saha & Cotta, 2006; Gould & Freer, 1983) with the consequence of reducing food produced by agriculture (Cherubini, 2010) and overexploiting, thus degrading, valuable productive soil (Hartemink, 2008). In order to limit this negative approach, bioethanol may be obtained from non-food biomasses which are rich in lignocellulosic material (Parrish & Fike, 2005; Sanerson et al., 2007), and easily grown on marginal soils (Fahd et al., 2012). Examples of such biomasses for second generation bioethanol are the perennial rhizomatous herbaceous plants, such as miscanthus (*Miscanthus χ Giganteus*, Greef et Deuter) in cool and humid areas, and giant reed (*Arundo donax*, L.) in warm and dry zones. Both these grasses show high productivity and favourable energy balance even at low nutrient and energy inputs (Lewandowski et al., 2000; Heaton et al., 2004a, Angelini et al., 2005). Therefore, the cropping of both grasses appear to be more ecologically convenient than annual food crops, since it needs no sowing and tillage, and, thus, reduces erosion risk (Heaton et al., 2004b) and improves soil carbon content and biodiversity.

Different methods have been devised to obtain bioethanol and other chemicals from lignocellulosic biomasses (Harmsen et al., 2010). However, once the cellulose has been separated,

the lignocellulosic residue is usually burnt or discarded (Cherubini, 2010), thereby preventing a mostly aromatic and precious photosyntate to be more profitably exploited. One technique separates cellulose from the bulk of lignocellulose at acidic pH by commonly employing inorganic acids such as sulphuric (H_2SO_4) and hydrochloric (HCl) acids (Sun & Cheng, 2002). This method yields easily fermentable sugars, although it also produces furfural, which is an inhibitor of microbial growth (Kumar et al, 2009). Even though this technique employs hazardous chemicals and is not cheap, it is still of practical interest (Clausen & Gaddy, 1993; Matsushita et al., 2011).

Another method has been developed in the paper-making industry and employs hydrogen peroxide (H_2O_2) at alkaline pH, as an alternative to environmentally hazardous chlorine and chlorine-based reagents (Sjöström, 1993). The use of strong bases, such as NaOH or $Ca(OH)_2$ is more environmentally compatible, and, in addition, provides an efficient delignification of fibrous cellulose and large solubility of separated lignin (Beckman et al. 1923). Lignin hydrolysis is significantly improved by adding the H_2O_2 oxidant to the alkaline reaction mixture (Gould, 1984). This alkaline oxidative solution easily disrupts cell walls, dissolves hemicellulose and lignin by hydrolysing uranic and acetic acid esters, and yields low crystalline cellulose (Yamashita et al., 2010). Moreover, α-aryl ether linkages in lignin phenolic units are readily cleaved and the conversion of the resulting phenolic units into, first, quinone methide intermediates, and, then, fragmented hydroxyl-phenolic groups, contributes to lignin solubilisation in alkaline solution (Chakar et al., 2004). Lignin is then easily recovered by lowering the pH (Sun et al., 2000). The resulting lignin seems to be lower in molecular weight and richer in carbonyl and carboxyl functionalities than Kraft or sulfite lignin (Holladay et al., 2007).

The objective of this work was to compare the physical-chemical characteristics of lignin obtained by an oxidative alkaline procedure with those of lignin extracted by sulfuric acid, from three different lignocellulosic biomasses: miscanthus, giant reed and a microbially pre-treated giant

reed. The isolated lignin was characterized by Attenuated Total Reflectance Infrared Fourier Transform spectroscopy (ATR-IR), solid state NMR spectroscopy (^{13}C-CPMAS spectra and T1ρH relaxation times), thermogravimetric analysis (TGA), and high performance size exclusion chromatography (HPSEC).

Material and Methods

Biomasses. Miscantus (*Miscanthus χ Giganteus*, Greef et Deuter) sample was provided by Phytatec Ltd (Birmingham, UK), whereas giant reed (*Arundo donax*, L) sample came from the University of Naples "Federico II" experimental farm at Bellizzi (Salerno, Italy). A third lignocellulose material from giant reed, that had been already undergone a microbial treatment for cellulose release, was provided by the Mossi and Ghisolfi Group of Tortona (Alessandria, Italy). From now on, miscanthus, raw giant reed and the pre-treated giant reed will be referred to MG, AD and RL, respectively.

Sulfuric acid hydrolysis. Lignocellulosic samples were treated with H_2SO_4 to remove cellulose as proposed by Cheshire & Mundie (1966). Briefly, 1 g was treated with 30 mL of 12 M H_2SO_4 solution for 16 hours at room temperature and under stirring. Then, water was added to the suspension to lower the H_2SO_4 concentration down to 1 M and kept continuously stirred for 3 hours at 105°C. The resulting lignin residue was centrifuged at 10,000 rpm for 30 min, dialyzed against deionized water, freeze-dried and stored in dried conditions before further analysis. The lignins obtained from RL, AD and MG cellulosic materials will be designated as SAL-RL, SAL-AD and SAL-MG, respectively.

Alkaline oxidative hydrolysis. Five grams of every sample was placed in a 150 mL of distilled water containing a 2 % H_2O_2 (v/v) solution. Then, the mixture pH was raised to 11.5 with a

4 M NaOH solution and stirred at 50°C. After 24 hours, the reaction mixture was centrifuged (10,000 rpm x 10 min). The pH of the supernatant (lignin and hemicellulose) was lowered to 5.5 and then 3 volumes of ethanol were added to flocculate the hemicellulose. The mixture was then filtered through a cellulose filter (Carlo Erba). The ethanol in the filtrate was removed by roto-evaporation and the pH lowered to 1.5-2.0 with 6 N HCl to precipitate the lignin. The lignin was then freeze-dried and stored in dried conditions for further analyses. The lignins obtained from RL, AD and MG will be referred to as Ox-RL, Ox-AD and Ox-MG, respectively.

Klason lignin determination. Lignin content in raw biomasses was determined by using the Klason assay as by the ASTM Standard method E 1721 (ASTM, 1998). Briefly, 300 mg of each sample were treated with 3.0 mL of 72% H_2SO_4 at 30°C for 2 h. The hydrolysed material is then transferred to a glass bottle and the acid was diluted until 4% concentration. Bottles were autoclaved in these sealed bottles for 1 h at 121°C. After completion of autoclave cycle, samples were filtered through a filtering crucible and dried at 105°C for 2 h until constant weight is reached. Finally, the crucibles containing the acid-insoluble lignin and the acid-insoluble ash are placed in a muffle furnace and ignited at 575±25°C for at least 3 h. Heating rate of 10°/min has been used to prevent flaming. The weight of crucibles with only acid-insoluble ash is recorded to the nearest 0.1 mg. Klason lignin (also called the acid-insoluble lignin) content was determined as follows

$$\%\,\text{lignin} = \frac{W2 - W3}{W1 \times \frac{T_{105}}{100}} \times 100 \qquad (1)$$

where

W_1: initial sample weitght

W_2: weight of crucible + acid-insoluble lignin + acid-insoluble ash

W_3: weight of crucible + acid-insoluble ash

$T_{105}/100$: % of total solids determined at 105°C.

All analyses were conducted in duplicate

Solubility assay. The solubility of lignins extracted was evaluated by placing 0.1 g of sample in 100 mL of distilled water and titrating the solution to pH 7 with 0.1 N NaOH. The solid residue remaining after titration was separated by centrifugation (10,000 rpm x 10 min), dried in oven (40°C) and weighted. The amount of soluble material was obtained by subtracting the weight of the residual pellet from that of initial lignin.

High performance size exclusion chromatography (HPSEC). The HPSEC system was composed by a Shimadzu LC-10-AD pump equipped with a Rheodyne rotary injector and 100-μL sample loop and two detectors in series: a UV/VIS detector (LC295, Perkin-Elmer, wavelength set at 280 nm) and a Refractive Index (RI) detector (Refractomonitor IV, Fisons Instrument). Since the UV-detector only gives information about the presence and the amount of chromophores and not about the compounds present in a mixture, a RI detector was also used. Even if it is much less sensitive then UV detectors, the RI response is more closely correlated to the concentrations of the eluted compounds and detects every eluted molecule, without regard to its chemical composition (Bidlingmeyer, 1993). The chromatographic column was a Biosep SEC s2000 300 x 7.80 mm (Phenomenex, USA), proceeded by a Biosep S-2000 35 x 7.80 pre-column (Phenomenex, USA) and a 2 mm inlet filter. The elution flow rate was set to 0.6 mL min^{-1} for an eluting solution made of 0.1 M NaH_2PO_4 solution, buffered at pH 6.5 with 0.1 M NaOH, The eluent was added with NaN_3 up to 0.3 g L^{-1} and filtered through 0.45 μm Millipore filter. A Unipoint Gilson Software was used to record each chromatographic run. The column calibration was obtained with the following sodium polystyrene sulfonates of known molecular masses: 123,000, 16,900, 6,780 and 1,200 Da. Ferulic acid (194 Da) and catechol (110 Da) have been also used as standards for the lowest molecular weights. The calibration curves provided the following relations between molecular weight (MW) and retention time (RT): log MW = -0.14 * RT + 6.339, for the UV detector, and, log

MW = -0.138 * RT + 6.338, for the RI detector. The coefficient of determination (R^2) for both curves was 0.97. Weight-average molecular weight (M_w), number-average molecular weight (M_n), and polydispersity ($P = M_w/ M_n$) were calculated as described elsewhere (Yau et al., 1979).

Statistical analysis. Statistical analysis (four replicates per each treatment) was conducted by applying the Kruskal-Wallis one-way analysis of variance test (Kruskal & Wallis, 1952) at the p-value less than or equal to 5%. This test is the nonparametric alternative to one-way ANOVA. It means that the null (H_0) and the alternative (H_1) hypothesis for this test are:

H_0: the populations ALL have the same medians;

H_1: the populations DO NOT ALL have the same medians.

Thermal analysis. Thermogravimetric analysis (TGA) and differential scanning calorimetry (DSC) curves were obtained by air combustion of sample (5-10 mg) in a simultaneous thermal analyzer (STA 6000-Perkin Elmer). The initial and final temperatures were 30° and 700°C, respectively, with an increasing rate of 10°C/min.

Attenuated Total Reflectance Infrared Fourier Transform spectroscopy (ATR-IR). The ATR-IR spectra were recorded with a Perkin Elmer 1720-X FT-IR spectrometer, equipped with a Perkin-Elmer Attenuated Total Reflectance accessory, accumulating up to 8 scans with a resolution of 4 cm^{-1}.

Solid state Nuclear Magnetic Resonance (NMR) spectroscopy. The ^{13}C-NMR spectra of the different lignins were recorded by Cross Polarization Magic Angle Spinning (CPMAS) solid-state spectroscopy with a Bruker AVANCE 300 using a rotating speed of 13±1 kHz, a recycle time of 2 s, an acquisition time of 33 ms and 4,000 scans and a contact time of 1 ms. A ^{1}H ramp sequence was used to account for possible inhomogeneity of the Hartmann-Hahn condition. The

free induction decays (FID) were transformed by applying a 4k zero filling and an exponential filter function with a line broadening of 100 Hz. Spectra processing was conducted by the 4.9.9.9 Mestre-C software. Proton spin–lattice relaxation times ($T1\rho(H)$) were measured and calculated by using the following equation:

$$I_{(t)} = I_0 \exp[-t_{SL}/ T1\rho(H)] \qquad (2)$$

where t_{SL} is the variable spin lock time, $I_{(t)}$ is the signal area at t^{th} spin lock time, and I_0 is the signal area in absence of any relaxation. The t_{SL} values were comprised between 10 and 10,000 ms.

Results and Discussion

The gravimetric results for the isolated lignins are reported in Table 1. The relative amount of Klason lignin for MG plants (26.5%) is comparable with those reported in literature (Villaverde et al., 2009; Brosse et al., 2009), whereas a slightly greater yield is shown by the analysis of AD (28.72%), as compared to the results usually found for this biomass (Shatalov & Pereira, 2002; Shatalov & Pereira, 2005; Scordia et al., 2011). This difference may be related to the delayed harvesting season used in this work for AD crops, which affects the relative composition of plant biomasses (Pirozzi et al., 2010). The winter harvesting is commonly adopted to promote the leaf shedding, in order to partly counteract the soil nutrients removal (Fagnano et al, 2010). Since the bio-synthesis of lignin in giant reed is closely dependent upon the plant maturation stage, the extension of crop cycle leads to an increase in the amount of lignin (Pascoal Neto et al., 1997). Moreover, the lowering of moisture content during winter period, allow the successive drying stage to be avoided (Cosentino et al., 2006), thereby further improving the yield of dry biomasses.

Lastly, the amount of acid-insoluble lignin released by RL residues (Table 1) is even larger than the one found in AD plants. This result may be associated with the former enzymatic attack to

which the RL sample was subjected: in fact, this pretreatment had the purpose to dissolve and solubilize the cellulose components, thus increasing the lignin concentration of the substrate.

A larger lignin content is shown by the SAL-extracts for all the three substrates, as compared to the corresponding Klason residues (Table 1). The possible condensation of cell wall carbohydrates and lignin molecules during acid hydrolysis (Dence, 1992), combined with the incorporation of sulfate into the lignin macrostructure, could have raised the final amounts.

Conversely a lower lignin recover has been obtained by the alkaline extraction process, which final products are less than 30% of the Klason lignin for all the samples (Table 1). The gravimetric data show that the alkaline extracted lignin is about the 6.33%, 5.21% and 10.47% of the initial weight for Ox-MG, Ox-AD and Ox-RL respectively, thereby suggesting a lower effectiveness of the alkaline extractive method. A possible explanation is the occurrence of ring opening during the reaction of lignin with H_2O_2 at alkaline pH, with the consequent yield of open-chain organic acids (Kadla et al., 1999). Since these compounds are still water-soluble even at pH 2.0, it is conceivable that an aliquot of reaction products may have been left in the supernatant after the acidification step. Moreover a portion of lignin-hemicellulose bonds may have not been fully hydrolyzed during the reaction and, therefore, some lignin components were flocculated and filtered away together with hemicelluloses, as the pH was lowered to 5.5.

The depolymerization and the oxidation extents of the lignin derivatives extracted by the SAL and Ox methods have been assessed by a solubility test (Table 2) carried out at neutral pH. While more than the 85% of Ox-lignin derivatives are easily dissolved, only the 5% of SAL samples have a noticeable solubility in water solution.

The lower solubility of SAL samples can be associated to the limited depolymerization activity achieved with the acid hydrolysis, coupled with the occurrence of condensation reactions involving carbohydrates and lignin (Dence, 1992), as indicated by the results of gravimetric

analyses (Table 1). On the contrary, the presence of the hydroperoxide anion promotes the oxidation of lignin (Kadla et al., 1997), with a considerable yield of oxidized aromatic compounds (Kadla et al., 1999). All the Ox-lignins from the three different materials showed a fast and homogenous dissolution behaviour even before the attainment of the optimum pH 7. The Kruskal-Wallis test indicates that there is no significant difference between al least one pair of medians (probability associated to the H-statistics = 0.44). The high oxidation extent of lignin extracted by H_2O_2 and NaOH is reached by both a saponification and a Dakin-like reaction (Kadla et al., 1999). The first reaction is caused by the interaction of ester groups with hydroxyl ions deriving from NaOH; the latter reaction consists of an oxidation of quinone methide (a well-known reaction intermediate formed in alkaline conditions) with the hydroperoxide anion. Both these reactions result in the oxidation of aromatic lignocellulosic constituents via a depolymerization of lignin.

High performance Size Exclusion Chromatography. The size of the three alkaline oxidized lignins has been analyzed by size exclusion chromatography. The UV chromatograms are reported in Figure 1, while the weight average molecular weight (M_w), the number average molecular weight (M_n) and polydispersity (P) for the UV-detected chromatograms are shown in Table 3. As can be noticed, the three compounds have a similar chromatographic behaviour, revealing the appearance of three different molecular-sized regions, including a broad region between 10 and 17 mL of elution volume, a sharp and intense peak at 18 mL and a final and small signal at 18.5 mL (Figure 1). The first chromatographic area comprises high molecular weight molecules, present in low amount, whereas both the second and the third peaks are attributed to lower molecular weight components. With respect to the high molecular weight region, Ox-RL shows both the highest M_w and M_n, followed by Ox-AD and then Ox-MG (Table 3). The values of polydispersity (P) indicate the non-polymeric nature of the molecules eluted within this first elution volume range for all the samples. In particular, the highest P level of Ox-MG (6.2) is related to the broadness of the earlier elution region (Figure 1), which might contain a large heterogeneous group of molecules.

Conversely the compounds eluted in the second and in the third peaks have a comparable hydrodynamic behavior for all the three samples, as indicated by the results of M_w and M_n calculation for all the Ox-lignins (Table 3). The calculated P data equals 1.0 for all the three biomasses, thus indicating that all the molecules in the fractions have the same hydrodynamic radius. In fact, the polydispersity is a measure of the heterogeneity of the molecules contained in a mixture. The closer to 1 is the P, the more homogeneous the sizes of the particles composing the mixture in exam are. The RI-detected chromatograms are reported in Figure 2. They clearly show that the amount of molecules in all of the three samples is practically equal for both the peaks around 12 mL and 18 mL in all the samples. The first and the second RI-detected peaks correspond to the first and the second regions in the UV chromatograms, respectively. The third peak in UV-detected chromatogram, instead, does not give any change in the refractive index of the eluent, maybe because of a very low concentration. A very interesting result is related to the M_w and M_n values calculated from the RI measurements (Table 3). They are not only comparable for the three Ox-samples, but also have a P approaching 1, indicating the monodisperse nature of the eluted compounds.

Thermal analyses. The TGA curves for both the SAL- and the Ox-samples are shown in Figure 3. The lignins extracted by sulfuric acid from AD and RL biomasses have a very close thermal behaviour, while the one isolated from MG shows a greater rate of weight loss up to approximately 400°C, then its thermal decay becomes comparable to the degradation of the other two biomasses. Instead, all the three samples coming from the alkaline oxidative hydrolysis appear to have similar decomposition up to 350°C and then Ox-MG seems to be degraded more slowly compared to Ox-AD and Ox-RL. In Figure 4, the derivatives of the TGA curves (DTG) are shown. The peak around 200°C for SAL-MG is attributed to the decomposition of lignin lateral chain (Brebu & Vasile, 2010), whereas the ones around 260°C in the DTG curves of Ox-AD and Ox-MG arise from the degradation of both lignin lateral chain and hemicellulose (Martins et al., 2003;

Hoareau et al., 2004; Brebu & Vasile, 2010). Furthermore, the peaks around 320°C may be ascribed to both the cleavage of C-C bonds between the lignin monomers (Graham & Mattila, 1971) and the evaporation of phenols (Sun et al., 2001). Finally, the signals around 400-500°C may be related to the aromatic ring decomposition (Yang et al., 2007).

In Figure 5, the results of the DSC analysis for the SAL- and Ox-lignins are reported. The exothermic peaks around 320°C may be related to the energy released from the cleavage of the lignin inter-unit linkages (Martin et al, 2010). Instead, the peak around 450-480°C may be attributed to both lignin and char volatilization (Wottitz et al., 2001). The analysis of the DSC curves for the H_2O_2-treated biomasses also reveals the presence of hemicellulose attributed to the peak around 280°C (Martin et al, 2010), thus confirming the nature of the peak at 260°C in the DTG curves. According to the results of the thermal analyses, lignin extracted by hydrogen peroxide is not completely pure, due to the presence of some residual carbohydrates, whereas the plant cell wall sugars are completely hydrolyzed during the treatment with sulfuric acid. This hypothesis is also supported by the ^{13}C NMR spectra (Figure 7) discussed below.

ATR-IR spectra. The infrared spectra for the lignins extracted by either sulfuric acid or hydrogen peroxide are shown in Figure 6. The peak around 3300 cm^{-1} has been attributed to the hydroxyl vibrations, whereas the signals around 2900, 2800 and 1460 cm^{-1} arises from to the presence of alkyl groups in the sample. It is noticeable that despite the peaks around 2900 and 2800 cm^{-1} are almost insignificant, there is a consistent signals at 1421 cm^{-1}, usually attributed to alkyl chain bending vibrations (Silverstein et al., 2005). This apparent incongruity is justified from the fact that the aromatic carbons also absorb at that frequency (Jahan et al., 2007), thus resulting in an increased amplitude for the signal around 1420 cm^{-1}. The signal around 1730 cm^{-1} may be related to the C=O stretching vibrations of either unconjugated ketone or carbonyl or ester groups (Boeriu et al., 2004), whereas the peak around 1370 cm^{-1} may be related to the O-H in-plane deformation of

alcohols and phenols and the one at 1210 cm^{-1} is originated by the C-O vibrations of primary alcohols. Indeed, the aromatic molecule vibrations are located around 1600, 1590, 1500, 1330, 1260, 1120, 1030 and 800 cm^{-1} (Hergert, 1971; Nada et al., 1998). More specifically, the peaks around 1590 cm^{-1} are attributed to the aromatic skeleton vibration plus the C=O stretching, whereas the absorption at 1328 cm^{-1} arises from the vibration of condensed syringyl and guaiacyl rings bound via position 5 (Jahan et al., 2007). The signals around 1260 and 820 cm^{-1} may be related to the guaiacyl ring breathing and to the out of-plane C-H vibration in guaiacyl units (C2, C5 and C6), respectively (Thring et al., 1990), while the one at 1123 cm^{-1} which may arise from the syringyl ring breathing vibration (Faix, 1992). Furthermore, the peak at 1030 cm^{-1} is attributed to the aromatic C-H in-plane deformation plus C–O deformation in primary alcohols plus unconjugated C–H stretching (Faix, 1992). Ether linkages are revealed by the peak at 1151 cm^{-1} (Silverstein et al., 2005). Finally, the peak around 1108 cm^{-1} has been attributed to the incorporation of sulfate into the molecular structure of the extract (Silverstein et al., 2005). This interpretation is also corroborated by some studies wherein lignosulfonates were studied by means of infrared spectrometry (Mollah et al., 1999; Singh et al., 2001).

The comparison of the spectra recorded for Ox-lignins show a better resolution than the ones obtained by acidic hydrolysis. This can be due to a more homogeneous sample collected after the alkaline hydrolysis. This higher homogeneity allowed us to gain more information about the structure of the extracted lignin, because of a much well resolved fingerprint region of the spectrum.

^{13}C-CPMAS- NMR spectra. The solid-state NMR spectra are reported in Figure 7. The resonance of carboxyl groups is located around 170 ppm. The peak at 152 ppm arises from the resonance of C3 and C5 in etherified syringyl units, whereas C3 and C4 in etherified guaiacyl units resonate around 148 ppm (Akim et al., 1993). The signal around 133 ppm has been assigned to the C1 in both syringyl and guaiacyl monomers (Wikberg & Maunu, 2004). Moreover, the peak around

127 ppm arises from the resonance of the carbons in lignin lateral chain (Robert, 1992). The signals at 112 and 116 ppm are due to the C2 and C5 of the guaiacyl units, respectively (Akim et al., 1993), whereas the one at 104 ppm is attributed to residual carbohydrates in the lignin fraction (Sun & Sun, 2002). Signals around 82 and 72 ppm are typical of alcoholic carbons and could be assigned to hydroxylated C-α and *C*-β in α-O-4/β-O-4 linkages in guaiacyl and syringyl units, respectively (Robert, 1992). Finally, the signal at 56 ppm arises from the resonance of the carbon in methoxy groups (Thring et al., 1990), whereas the signals around 39, 32 and 29 ppm are due to alkyl carbons.

The absence of signals attributable to carbohydrates in the spectra of SAL-lignins clearly shows the effectiveness of hydrolysis of this extractive method, thus confirming the results shown by the thermal analyses and the infrared spectrometry. The Ox-lignin spectra show a higher spectral resolution than the spectra recorded for SAL-lignins, thus indicating a higher homogeneity of the material.

Relaxation times (T1ρH) as measured by solid-state NMR spectroscopy. In order to make assumptions about the mobility of the lignins extracted, the proton relaxation time in the rotating frame (T1ρH) was calculated (Table 1). The relaxation parameter T1ρH is measured via the cross-polarization technique and it is used to characterize polymer dynamics in the kilohertz range (within the 10-100 kHz range). The kHz regime motions are associated with cooperative polymer backbone rearrangements involving the collective movements of a large number of monomer units (Boyer et al., 1968; Connor, 1971). The T1ρH values are also correlated to the rate of the spin diffusion. This is, in turn, inversely related to the proton–proton intermolecular distance and, by this, to the degree of molecular motions (Sozzani, 1993). This T1ρH reflects the strength of ^{1}H-^{1}H dipolar interactions and indicate whether the various protons belong to a common spin reservoir. Thus, the observed T1ρH is an average over all the protons in the sample, as a result of spin diffusion (McCall, 1971). Hence, the T1ρH experiment provides valuable structural information, because the measured values

depend on whether two protons of a solid material are sufficiently rigid and close in space to be "intimately" mixed (Stejskal et al., 1981). This means that the T1ρH can be used as an indication of the rigidity of a group of molecules.

The T1ρH values are longer for all the SAL- than the Ox-lignins, indicating that SAL-samples have a lower mobility than the Ox-materials. The values for the samples collected after alkaline hydrolysis may be related to a higher proton density around the carbon atoms (Duer, 2002), which leads to a faster relaxation in the rotating frame, due to the higher flexibility of the molecules extracted by alkaline hydrolysis. Various authors reported a correlation between the T1ρH values and the mobility of the plant cell wall. Fenwick et al. (1996) examined the rigidity of tomato cell wall and found that it is positively correlated to the T1ρH, namely, the longer the T1ρH, the more rigid the plant cell wall components. Also, Fenwick et al. (1997) studied growing and nongrowing celery collenchyma by solid-state NMR relaxometry *in vivo* and reported a shorter T1ρH for growing, more mobile tissues, whereas nongrowing, less mobile cells exhibited longer T1ρH. Indeed, Gil et al. (1997) analysed the mobility of cell wall components before and after the removal of suberin and found that the desuberization caused an increase in cell wall flexibility, thus justifying the decrease in the T1ρH. These studies corroborate the hypothesis that the Ox-lignins are well depolymerized, compared to SAL-ones, also confirming the validity of the alkaline extractive method as a technique to produce a lignin primarily made of low molecular weight compounds.

Conclusions

Since the lignin recovered by biorefineries is attracting always more the interest of both scientific and industrial world, it is important to study it at a molecular level. In this study, lignin from three different biomasses (miscanthus, giant reed and a lignocellulosic residue from giant reed) has been extracted with either H_2SO_4 or H_2O_2 at alkaline pH. Then, we examined its molecular properties by means of various analytical methods.

From gravimetrical data, it seems that the Ox method is not very efficient, due to the low extraction yields. This could be caused by either the formation of many low-molecular weight open chain organic acids still soluble at very acidic pH or to the missed optimization of the process for the materials used in this study. The very high amount of lignin extracted by sulfuric acid, instead, may be due to both the condensation of some carbohydrates on the lignin structure and the incorporation of sulfate. Moreover, the solubility data show that the Ox-lignins are much more water-soluble than the SAL-lignins. This is probably due to a much lower depolymerization extent of the lignin isolated by sulfuric acid.

Thermogravimetric analyses reveal that the lignin isolated by alkaline hydrogen peroxide treatment still contains a certain amount of carbohydrates, while the one extracted by sulfuric acid do not contain sugars, thus being more efficient in hydrolyzing them, leaving, instead, only the plant cell wall aromatic components

The HPSEC chromatograms (recorded only for Ox-lignins) reveal the presence of large molecules together with much smaller molecules. These results show that the greatest number of molecules with high MW is contained in Ox-RL, but that the size of total molecules present in the three Ox-lignins are alike.

This study confirms IR and NMR spectroscopies as very helpful techniques to identify differences between various lignin isolation methods As a matter of fact, these spectroscopic techniques clearly reveal the more oxidized nature of the Ox-lignins compared to the SAL-ones and, also, that the sulfuric acid treatment is much more efficient in hydrolyzing the plant cell wall carbohydrates.

The T1ρH values are shorter for the Ox-samples than for the SAL-ones. Since the lower the T1ρH, the higher the mobility of lignin, it can be argued that Ox-materials are more efficiently depolymerized than the SAL-lignins.

These data point out that the sulfuric acid treatment results in a higher molecular size and an increased hydrophobicity of lignins. Conversely, the alkaline treatment leads to a well-depolymerized, hydrophilic lignin. Between the two extractive methods employed, the most interesting is surely the alkaline hydrogen peroxide treatment, which allows the isolation of a greatly oxidized lignin. This material is highly water-soluble and can be used in all the reactions involving water. Moreover, Ox-lignins can also be used as a dispersant or as binders (Fredheim et al., 2002; Groote et al., 1985). Furthermore, they could also be used as plant growth stimulators. This would result in a decrease of the inputs of organic fertilizers to the soil and, contemporarily, in an increase of the ecological and economical liability of the biorefineries.

Table 1. Percentage (%) with respect to the treated biomass of lignin separated by sulfuric acid (SAL), alkaline-H_2O_2 (Ox) and Klason method from different lignocellulose biomasses.

Biomass[a]	SAL	Ox	Klason
RL	43.80	10.47	36.15
AD	34.10	5.21	28.72
MG	35.55	6.33	26.50

a. RL= lignin from lignocellulose residue of giant reed; MG = lignin from miscanthus biomass; AD = lignin from giant reed biomass.

Table 2. Average percentage (%) of solubility in water at pH 7 and T1ρH-NMR values (ms) calculated for lignins separated by sulfuric acid (SAL) and alkaline oxidative (Ox) methods from different biomasses.

	Biomass[a]					
	RL		**MG**		**AD**	
Treatment	**SAL**	**Ox**	**SAL**	**Ox**	**SAL**	**Ox**
Solubility	5	88	3	9	4	85.5
T1ρH	11.85	7.27	8.42	7.99	13.26	9.8

a. RL= lignin from lignocellulose residue of giant reed; MG = lignin from miscanthus biomass; AD = lignin from giant reed biomass.

Table 3. Weight average (M_w) and number average (M_n) molecular weights and polydispersity (P) calculated from UV- and RI-detected HPSEC chromatograms for lignins separated by alkaline oxidation (Ox) from different biomasses.

	UV-detector				RI-detector			
Biomass[a]	**Region**[b]	**M_w**	**M_n**	**P**	**Region**	**M_w**	**M_n**	**P**
	A	2302	844	2.7	A	2925	2887	1.0
Ox-RL	B	132	130	1.0	B	164	163	1.0
	C	64	62	1.0	C[c]	-	-	-
	A	1541	664	2.3	A	2911	2870	1.0
Ox-AD	B	141	139	1.0	B	170	170	1.0
	C	70	67	1.0	C[c]	-	-	-
	A	1319	214	6.2	A	2952	2912	1.0
Ox-MG	B	151	148	1.0	B	167	166	1.0
	C	74	71	1.0	C[c]	-	-	-

a. Ox-RL= lignin from lignocellulose residue of giant reed; Ox-AD = lignin from giant reed biomass; Ox-MG = lignin from miscanthus biomass;

b. Interval of retention times in HPSEC chromatograms: A = 10-17 mL; B = 17.9-18.2 mL; C 18.4-18.6 mL;

c. refraction not present in chromatogram.

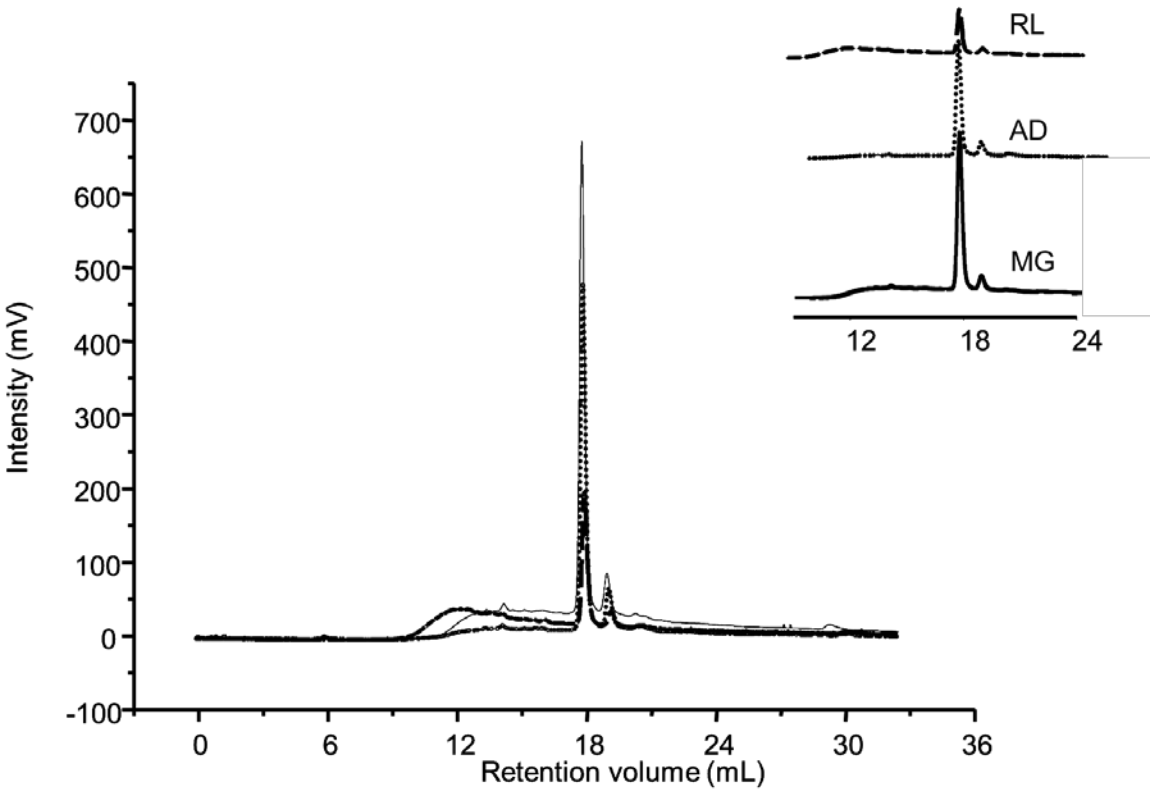


Figure 1. UV-detected HPSEC chromatograms for lignins for lignins separated by alkaline oxidation (Ox). RL: lignin from lignocellulose residue of giant reed; AD = lignin from giant reed biomass; MG = lignin from miscanthus biomass.

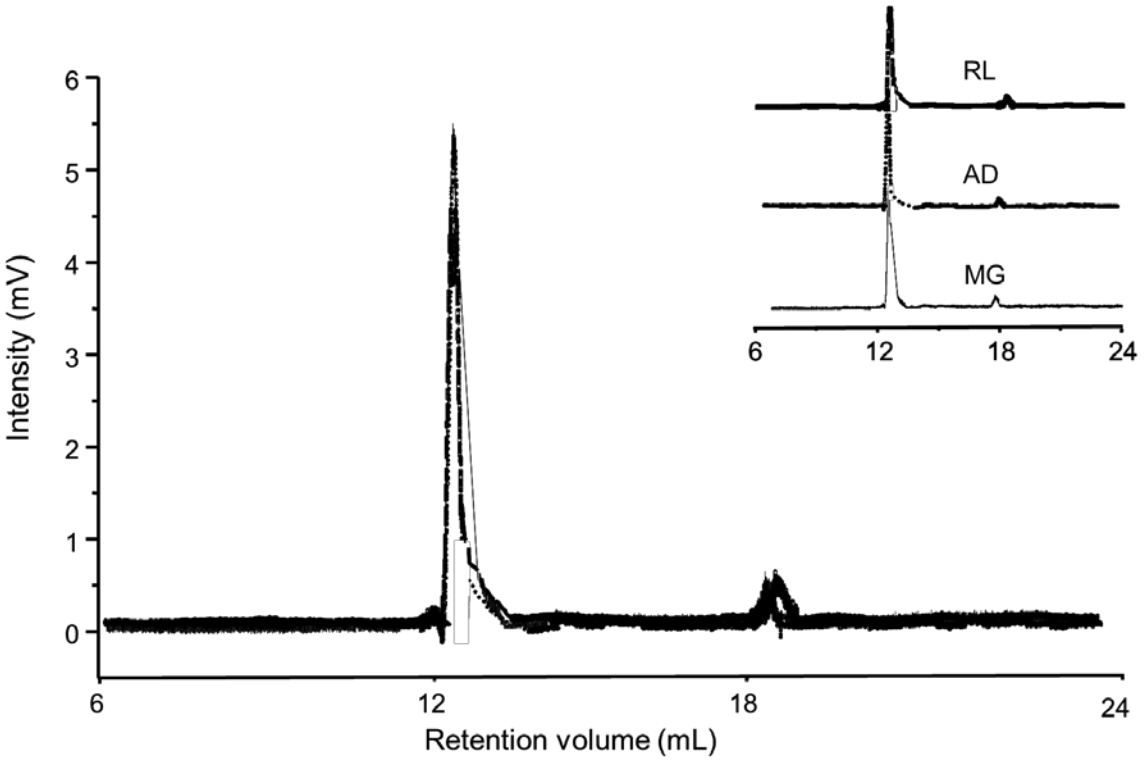


Figure 2. RI-detected HPSEC chromatograms for lignins for lignins separated by alkaline oxidation (Ox). RL: lignin from lignocellulose residue of giant reed; AD = lignin from giant reed biomass; MG = lignin from miscanthus biomass.

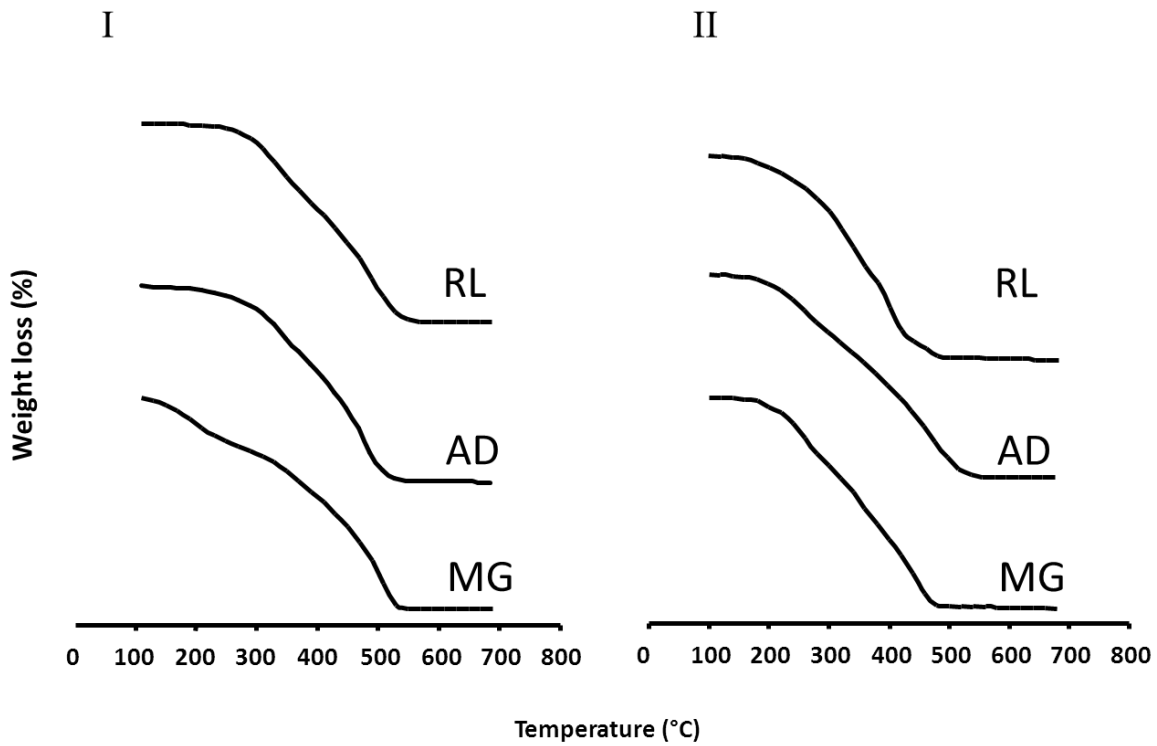


Figure 3. TGA curves for biomasses treated with sulfuric acid (I) and hydrogen peroxide (II). RL: lignin from lignocellulose residue of giant reed; AD = lignin from giant reed biomass; MG = lignin from miscanthus biomass.

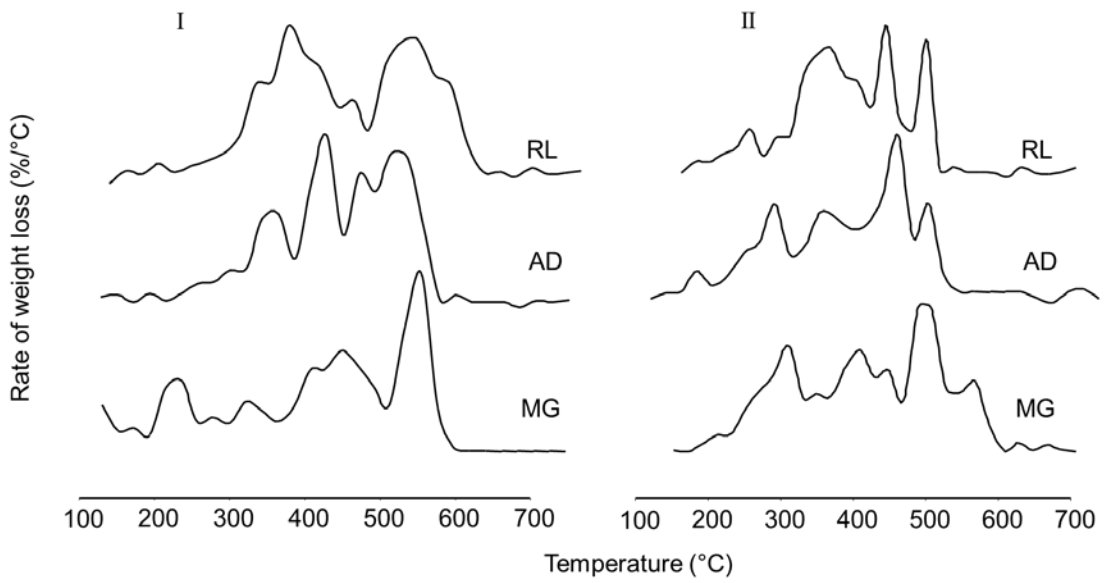


Figure 4. DTG curves for biomasses treated with sulfuric acid (I) and hydrogen peroxide (II). RL: lignin from lignocellulose residue of giant reed; AD = lignin from giant reed biomass; MG = lignin from miscanthus biomass.

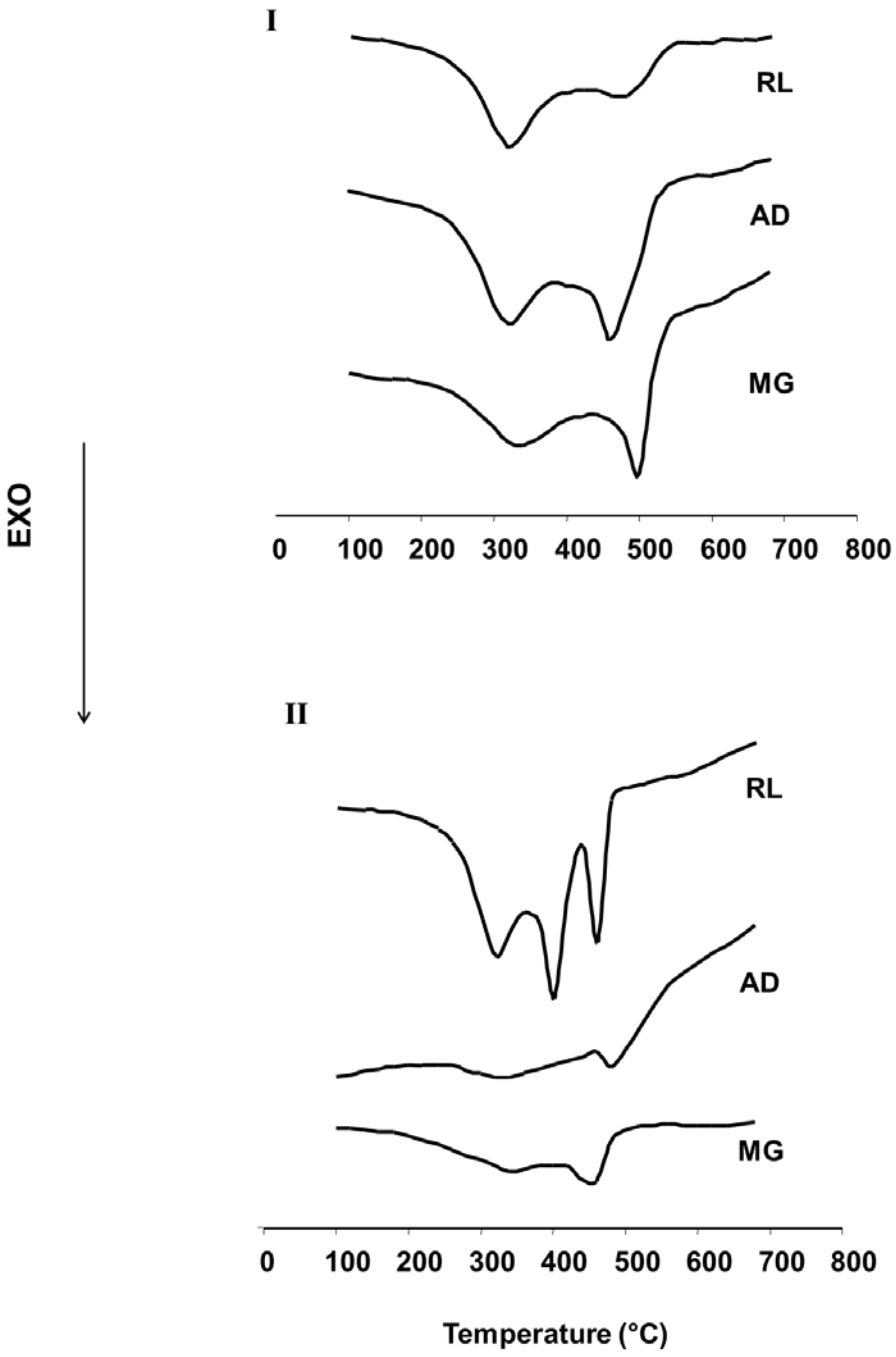


Figure 5. DSC curves for biomasses treated with sulfuric acid (I) and hydrogen peroxide (II). RL: lignin from lignocellulose residue of giant reed; AD = lignin from giant reed biomass; MG = lignin from miscanthus biomass.

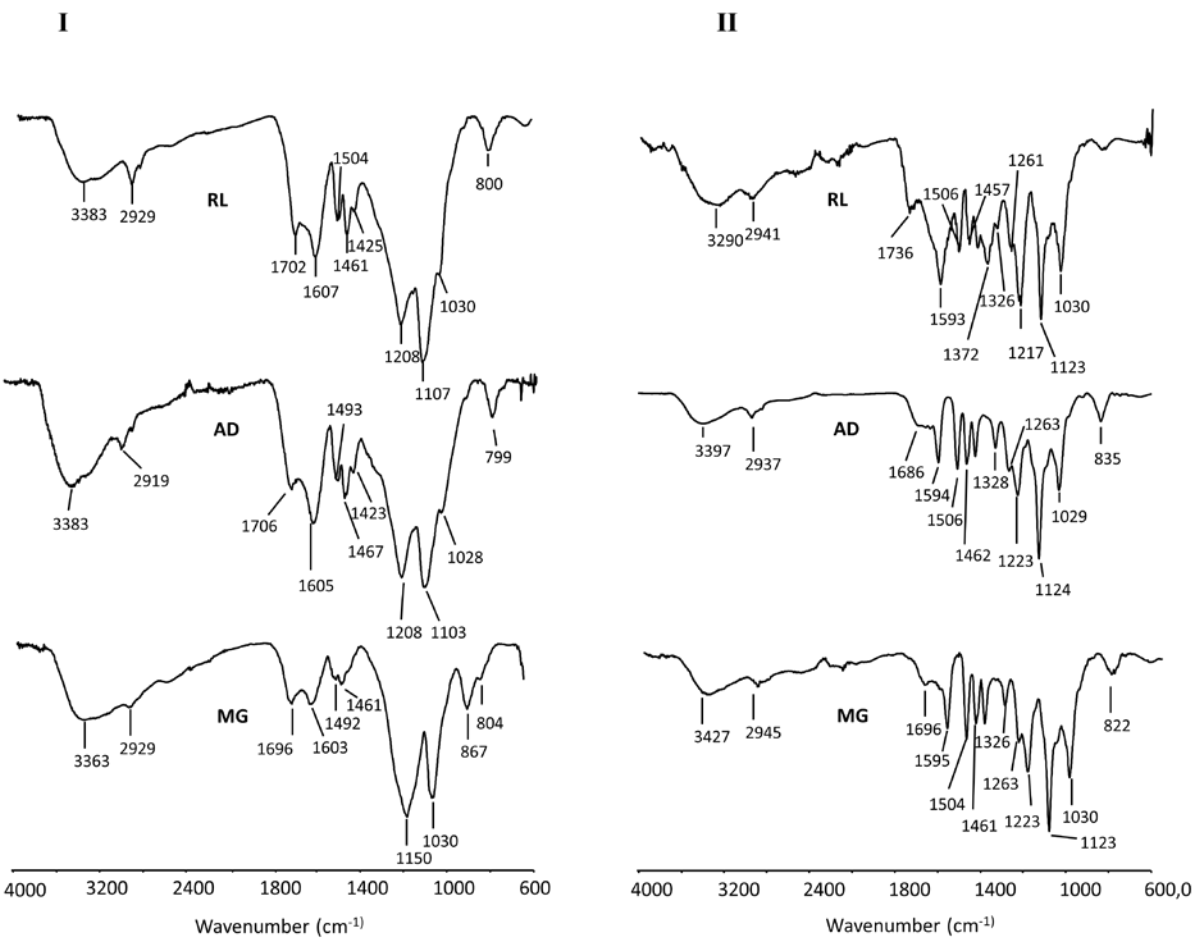


Figure 6. ATR-IR spectra for biomasses treated with sulfuric acid (I) and hydrogen peroxide (II). RL: lignin from lignocellulose residue of giant reed; AD = lignin from giant reed biomass; MG = lignin from miscanthus biomass.

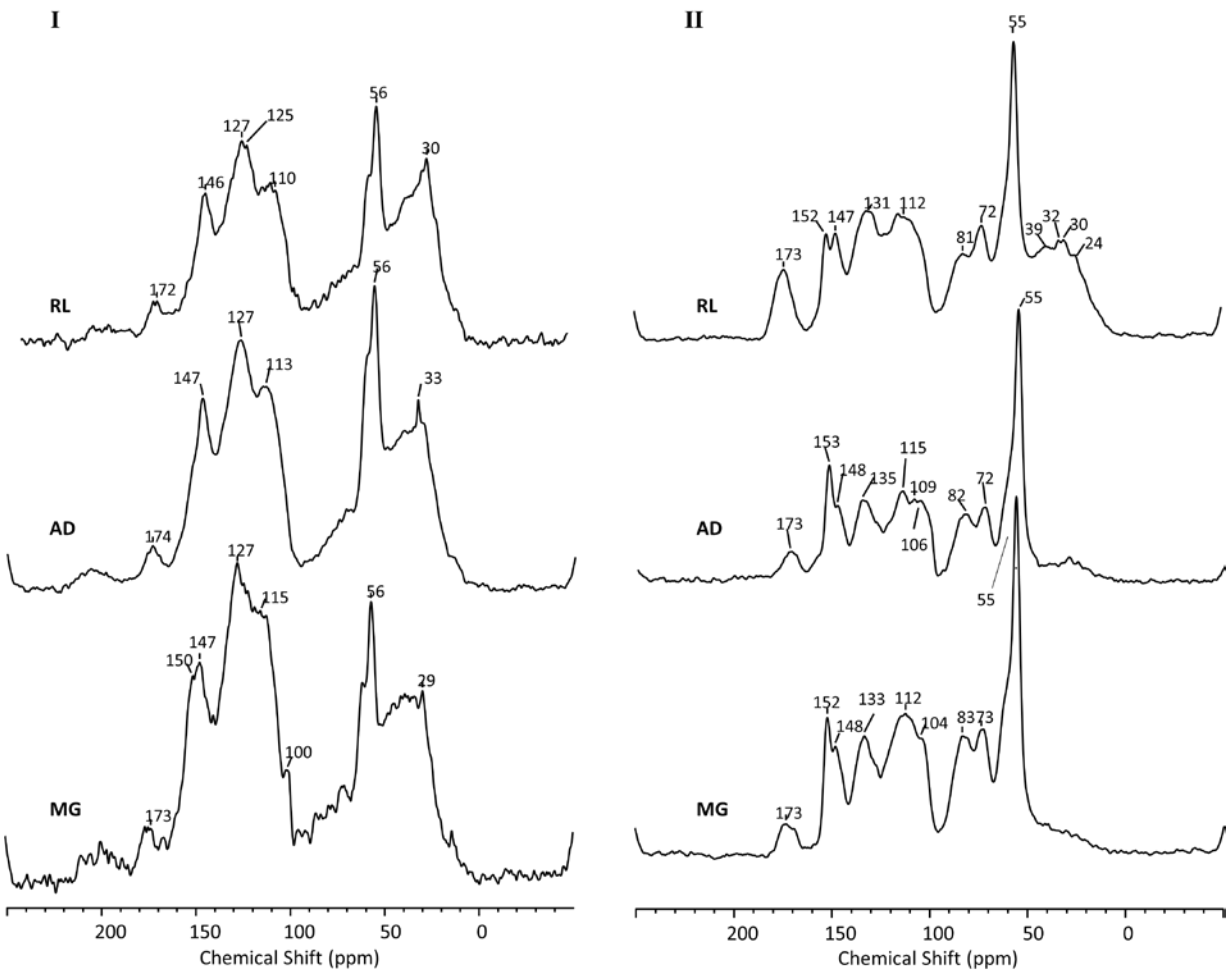


Figure 7. ^{13}C CPMAS NMR spectra for biomasses treated with sulfuric acid (I) and hydrogen peroxide (II). RL: lignin from lignocellulose residue of giant reed; AD = lignin from giant reed biomass; MG = lignin from miscanthus biomass.

References

Abdullah, S S; Yusup, S; Ahmad, M M; Ramli, A; Ismail, L (2010) Thermogravimetry Study on Pyrolysis of Various Lignocellulosic Biomass for Potential Hydrogen Production. World Academy of Science, Engineering Technology, 72. 129-133.

Akim, L G; Shevchenko, S M; Zarubini, M Y (1993) 13C NMR studies on lignins depolymerized with dry hydrogen iodide. Wood and Science Technology, 27, 241-248.

Angelini, L G; Ceccarini, L; Bonsri, E (2005) Biomass yield and energy balance of giant reed (*Arundo donax*, L.) cropped in central Italy as related to different management practices Europ. J. Agronomy 22, 375–389.

ASTM (1998), in Annual Book of ASTM Standards, ASTM Method E 1721-95, vol. 11.05, American Society for Testing and Materials, West Conshohocken, PA.

Beckmann, E, Liesche, O; Lehmann, F (1923) Qualitative und quantitative Unterschiede der Lignin einiger Holz- und Stroharten. Biochemische Zeitschrift, 139, 491–508.

Bidlingmeyer, B A. Practical HPLC Methodology and Applications. John Wiley & Sons, New York, NY, 1993, 83.

Boeriu, C G; Bravo, D; Gosselink, R J A; van Dam, J E G (2004) Characterisation of structure-dependent functional properties of lignin with infrared spectroscopy. Industrial Crop and Products, 20, 205-218.

Boyer, R F (1968) Dependence of mechanical properties on molecular motion in polymers. Polymer Engineering and Science, 8, 161-185.

Brebu, M & Vasile, C (2010) Thermal degradation of wood – A review. Cellulose Chemistry and Technology, 44, 353-363.

Brosse, N; Sannigrahi, P; Ragauskas, A (2009) Pretreatment of Miscanthus x giganteus Using the Ethanol Organosolv Process for Ethanol Production. Industrial & Engineering Chemistry Research, 48, 8328–8334.

Chakar, F S, Ragauskas, A J (2004). Review of current and future softwood kraft lignin process chemistry. Industrial Crops and Products. 20, 131–141.

Cherubini, F (2010) The biorefinery concept: Using biomass instead of oil for producing energy and chemicals. Energy Conversion and Management 51, 1412–1421.

Cheshire, M. V. & Mundie, M. (1966). “The hydrolytic extraction of carbohydrates from soil by sulphuric acid”. Journal of Soil Science, 17, 372-3814.

Clausen, E C; Gaddy, J L (1993) Concentrated sulfuric acid processing for converting lignocellulosic materials to sugars, U. S. Pat. 5,188,673.

Connor T M; NMR, Basic Principles and Progress, Vol. 4, P. Diehl, E. Fluck, and R. Kosfeld, Ed., Springer-Verlag, New York, N.Y., 1971.

Connors, W J; S Sarkanen, S; J. L. McCarthy, J L (1980) Gel chromatography and association complexes of lignin. Holzforschung 34, 80-85.

Cosentino, S L; Copani, V; D'Agosta, G M; Sanzone, E; Mantineo, M (2006) First results on evaluation of Arundo donax L. clones collected in Southern Italy. Industrial Crops and Products, 23, 212–222.

Dence C W; The Determination of Lignin. In Methods in lignin chemistry; Lin, S Y, Dence, C W, Eds.; Springer-Verlag; Berlin, 1992; pp 33-62.

Duer M J; Solid-state NMR spectroscopy: principles and applications. Blackwell Science, UK, 2002.

Fagnano M, Impagliazzo A, Mori M, Fiorentino N (2010) Produzione dell'*Arundo donax* in ambiente collinare campano. In: Atti XXXIX Convegno SIA (Mastrorilli M., ed.) Roma (Italia) 20-22 settembre 2010, 207-208.

Fahd, S; Fiorentino, G; Mellino, S; Ulgiati, S (2012) Cropping bioenergy and biomaterials in marginal land: The added value of the biorefinery concept. Energy, 37, 79-93.

Faix, O; Fourier Transfom Infrared Spectroscopy. In Methods in Lignin Chemistry; Lin, S. Y., Dence, C. W., Eds.; Springer- Verlag: Berlin, 1992; 81-109.

Faix, O; Lange, W; Salud, E C (1981) The Use of HPLC for the Determination of Average Molecular Weights and Molecular Weight Distributions of Milled Wood Lignins from *Shorea polysperma* (Blco.). Holzforschung, 35, 3-9.

Fenwick, K M; Jarvis, M C; Apperley, D C; Seymour, G B; Bird, C R (1996) P olymer mobility in cell walls of transgenic tomatoes with reduced polygalacturonase activity. Phytochemistry, 42, 301-307.

Fenwick, K M; Jarvis, M C; Apperley, D C(1997) Estimation of Polymer Rigidity in Cell Walls of Growing and Nongrowing Celery Collenchyma by Solid-State Nuclear Magnetic Resonance in Vivo. Plant Physiology, 115, 587-592.

Fredheim, G E, Braaten, S M, Christensen, B E (2002) Molecular weight determination of lignosulfonates by size-exclusion chromatography and multi-angle laser light scattering. Journal of Chromatography A, 942, 191–199.

Garside, P & Wyeth, P (2003) Identification of Cellulosic Fibres by FTIR Spectroscopy. Studies in Conservation 48, 269-275.

Gil A M; Lopes, M; Rocha, J; Pascoal Neto, C (1997) A ^{13}C solid state nuclear magnetic resonance spectroscopic study of cork cell wall structure-the effect of suberin removal. International Journal of Biological Macromolecules, 20, 293–305.

Gould J M & Freer, S N (1983) High-Efficiency Ethanol Production from Lignocellulosic Residues Pretreated with Alkaline H_2O_2. Biotechnology and Bioengineering, 26, 628-631.

Gould, J M (1984). Alkaline peroxide delignification of agricultural residues to enhance enzymatic saccharification. Biotechnology and Bioengineering, 26, 46-52.

Graham, G & Mattila, T High Energy Degradation. In "Lignin – Occurrence, Formation, Structures and Reactions", Sarkanen, K V; Ludwig, C H, Wiley, New York, 1971, pp. 575.

Groote, C R A M, Neumann, M G, Cerqueira, M, Mendonça, CC, Bernardo, R (1985). Brazilian Patent PI 8.300.948.

Harmsen, P F H; Huijgen, W J J; Bermúdez López, L M; Bakker, R R C. Literature Review of Physical and Chemical Pretreatment Processes for Lignocellulosic Biomass. ECN Biomass, Coal and Environmental Research 2010.

Hartemink, A E (2008) Chapter 3 Sugarcane for Bioethanol: Soil and Environmental Issues. Advances in Agronomy, 99, 125-182.

Heaton, E A;Clifton-Brown, J;Voigt, T; Jones M B; Long S P (2004a) Miscanthus for renewable energy generation: European Union experience and projections for Illinois. Mitigation Adapt. Strategies Global Change, 9, 433–451.

Heaton E, Voigt T, Long S P (2004b) A quantitative review comparing the yields of two candidate C4 perennial biomass crops in relation to nitrogen, temperature and water. Biomass and Bioenergy, 27, 21–30.

Hergert, H L (1971) Infrared Spectra, in Lignins: Occurrence, formation, structure and reaction; Sarkanen, K V, Ludwing, C H, Eds.; Wiley-Interscience; New York, 1971; pp 268-272.

Hoareau, W; Trindade, W G; Siegmund, B; Castellan, A; Follini, E (2004) Sugar cane bagasse and curaua lignins oxidized by chlorine dioxide and reacted with furfuryl alcohol: characterization and stability. Polymer Degradation and Stability, 86, 567-576.

Holladay, J E; Bozell, J J; White, J F; Johnson, D (2007). Top Value-Added Chemicals from Biomass Volume II—Results of Screening for Potential Candidates from Biorefinery Lignin. Pacific Northwest National Laboratory, U. S. Dempartment of Energy.

Jahan, M S; Nasima Chowdhury, D A; Khalidul Islam, M; Iqbal Moeiz, S M (2007) Characterization of lignin isolated from some nonwood available in Bangladesh. Bioresource Technology 98, 465-469.

Jakab, E, Faix O, Till F (1997) Thermal decomposition of milled wood lignin studied by thermogravimetry/mass spectrometry. Journal of Analytical and Applied Pyrolysis, 40 41, 171-186.

Kadla, J K; Chang, H, Jameel, H (1997) The Reactions of lignins with hydrogen peroxide at High temperature. Part I. The oxidation of lignin model compound. Holzforschung 51, 428-434.

Kadla, J K; Chang, H, Jameel, H (1999) The Reactions of lignins with hydrogen peroxide at High temperature. Part 2.The oxidation of kraft lignin. Holzforschung 53, 277-284.

Kaparaju, P; Serrano, M; Thomsen, A B; Kongjan, Angelidaki, I (2008) Bioethanol, biohydrogen and biogas production from wheat straw in a biorefinery concept. Bioresource Technology, 100, 2562-2568.

Kruskal, W H; Wallis, W A (1952) Use of Ranks in One-Criterion Variance Analysis. Journal of the American Statistical Association, 47, 583-621.

Kumar, P; Barrett, D M; Delwiche' M J; Stroeve, P (2009) Methods for Pretreatment of Lignocellulosic Biomass for Efficient Hydrolysis and Biofuel ProductionIndustrial & Engineering Chemistry Research 48, 3713–3729.

Lewandowski, I; Clifton-Brown, J C; Scurlock, J M O; Huisman W (2000) Miscanthus: European experience with a novel energy crop. Biomass and Bioenergy, 19, 209–27.

Li, J; Li, B: Zhang, X (2002), Comparative studies of thermal degradation between larch lignin and manchurian ash lignin. Polymer Degradation and Stability, 78, 279–285.

Majcherczyk, A & Hüttermann, A (1997) Size-exclusion chromatography of lignin as ion-pair complex, Journal of Chromatography, 764, 183-191.

Mancera, A; Fierro, V;Pizzi, A;Dumarçay, S; Gérardin, P; Velásquez, J; Quintana, G; Celzard, A (2010) Physicochemical characterisation of sugarcane bagasse lignin oxidized by hydrogen peroxide, Polymer Degradation and Stability 95, 470.476.

Martin, A R; Martins, M A; da Silva, O R R F, Mattoso, L H C (2010) Studies on the thermal properties of sisal fiber and its constituents. Thermochimica Acta, 506, 14-19.

Martins, M A; Joekes, I (2003) Tire rubber–sisal composites: Effect of mercerization and acetylation on reinforcement, Journal of Applied Polymer Science, 89, 2507-2515.

Matsushita, Y; Inomata, T; Takagi, Y; Hasegawa, T; Fukushima, K (2011) Conversion of sulfuric acid lignin generated during bioethanol production from lignocellulosic materials into polyesters with ε–caprolactone. Journal of Wood Science, 57, 214-218.

McCall, D W (1971) Nuclear magnetic resonance studies of molecular relaxation mechanisms in polymers. Accounts of Chemical Research, 4, 223-232.

Minor, J (1979) Gel Permeation Chromatography of Polymers from Wood. Journal of Liquid Chromatography, 2, 309.

Mollah, M Y A; Yu, W; Schennach, S; Cocke, D L (2000) A Fourier transform infrared spectroscopic investigation of the early hydration of Portland cement and the influence of sodium lignosulfonate. Cement and Concrete Research, 30, 267–273.

Nada, A A M A; Yousef, M A; Shaffei, K A; Salah, A M (1998) Infrared spectroscopy of some treated lignins. Polymer Degradation and Stability, 62, 157-163.

Parrish, D & Fike, J H (2005) The biology and agronomy of switchgrass for biofuels. Critical Reviews in Plant Science 2005, 24, 423-459.

Pirozzi D, Yousuf A, Fagnano M, Mori M, Fiorentino N, Impagliazzo A (2010) Lipids production by yeast grown on giant reed biomass. Journal of Biotechnology , 150, S1, 167-168.

Robert, D Carbon-13 nuclear magnetic resonance. In Methods in Lignin Chemistry; Lin, S. Y., Dence, C W, Eds.; Springer- Verlag: Berlin, 1992; pp 250-273.

Saha, B C & Cotta, M A (2006) Ethanol Production fron Alkaline Peroxide Pretreated Enzymatically Saccharified Wheat Straw. Biotechnology Progress, 22, 449-453.

Sanerson, M A; Adler, P R; Martin, N P; Biomass, energy, and industrial uses of forages. In Forages Volume II: The science of grassland agriculture, 6 th Ed.; Barnes, R F; Nelson, C J, Moore, K J; Collins, M, Eds.; Iowa State University Press: Ames, IA, 2007; pp. 635-647.

Sarkanen, S; Teller D C; Hall, J; (1981) Lignin. 18. Associative effects among organosolv lignin components. Macromolecules, 14, 426–434.

Scordia, D; Cosentino, S L; Lee, J W; Jeffries, T W (2011) Dilute oxalic acid pretreatment for biorefining giant reed (*Arundo donax* L.). Biomass and Bioenergy, 35, 3018-3024.

Shatalov, A A; Pereira, H (2002) Influence of stem morphology on pulp and paper properties of Arundo donax L. reed. Industrial Crops and Products 15, 77–83.

Shatalov, A A; Pereira, H (2005) Kinetics of organosolv delignification of fibre crop Arundo donax L. Industrial Crops and Products, 21 203–210.

Silverstein, R M; Webster, F X; Kiemle, D; Spectrometric Identification of Organic Compounds, Wiley, NY, 2005.

Singh, N B; Singh, V D; Rai, S; Chaturvedi, S (2002) Effect of lignosulfonate, calcium chloride and their mixture on the hydration of RHA-blended portland cement. Cement and Concrete Research, 32, 387–392.

Sjöström, E (1993). Wood Chemistry: Fundamentals and Applications. Academic Press. ISBN 012647480X. OCLC 58509724.

Skreiberg, A; Skreiberg, Ø; Sandquist, J;Sørum, L (2011) TGA and macro-TGA characterisation of biomass fuels and fuel mixtures. Fuel, 90, 2182, 2197.

Sozzani, P. in F. Morazzoni (Ed.), From Molecular Materials to Solids, Milan, 1993, pp 47–69.

Stejskal, E O; Schaefer, J; Sefcik, M D; McKay, R A (1981) Magic-angle carbon-13 nuclear magnetic resonance study of the compatibility of solid polymeric blends. Macromolecules, 14, 275-279.

Stewart, D (2008), Lignin as a base material for materials applications: Chemistry, application and economics. Industrial Crops and Products, 27, 202-207.

Sun, RC & Sun, X F (2002) Fractional and structural characterisation of hemicellulose isolated by alkali and alkaline peroxide from barley straw. Carbohydrate Polymers, 49, 415-423.

Sun, R C; Tomkinson, J (2002) Comparative study of lignins isolated by alkali and ultrasound-assisted alkali extractions from wheat straw. Ultrasoinic Sonochemistry, 9, 85-93.

Sun, R, Tomkinson, J, Wang, S; Zhu, W (2000). Characterization of lignins from wheat straw by alkaline peroxide treatment. Polymer Degradation and Stability 67, 101-109.

Sun, R; Lu, Q; Sun, X F (2001) Physico-chemical and thermal characterization of lignins from *Caligonum monogoliacum* and *Tamarix spp*. Polymer Degradation and Stability 72, 229–238.

Sun, Y; Cheng, J (2002) Hydrolysis of lignocellulosic materials for ethanol production: a review. Bioresource Technology 83 1–11.

Thring, R W; Chornet, E; Bouchard, J; Vidal, P F; Overend, R P (1990) Characterization of lignin residues derived from the alkaline hydrolydid of glycol lignin. Canadian Journal of Chemistry, 68, 82-89.

Villaverde, J J; Li, J; Ek, M; Ligero, P; De Vega, A (2009) Native Lignin Structure of *Miscanthus x Giganteus* and Its Changes during Acetic and Formic Acid Fractionation. Journal of Agricultural and Food Chemistry, 57, 6262–6270.

Walshaw, J; Mathison G W; Fenton, T F; Sedgwick, G; Hsu, H; Recinos-Diaz, G; Suleiman, A (1998) Characterization of forages by differential scanning calorimetry and prediction of their chemical composition and nutritive value. Animal Feed Science Technology, 71 309-323.

Wikberg, H & Maunu, S L (2004) Characterisation of thermally modified hard- and softwoods by ^{13}C CPMAS NMR. Carbohydrate Polymers, 58, 461-466.

Wottitz C. A., C. E. López Pasquali and H. A Herrera. “Thermal Decomposition of Algarrobo negro (Prosopis nigra). Wood and its main components: lignin, alpha cellulose and hemicelluloses "A" and B""- Latin American Applied Reseach 31, 37-40, (2001).

Yamashita, Y, Shono, M, Sasaki, C, Nakamura, Y (2010). Alkaline peroxide pretreatment for efficient enzymatic saccharification of bamboo Carbohydrate Polymers 79, 914–920.

Yang, H; Yan, R; Chen, H; Lee D H; Zheng, C (2007) Characteristics of hemicellulose, cellulose and lignin pyrolysis. Fuel, 86, 1781-1788.

Yau W W, Kirkland J J, Bly D D, eds. Modern size exclusion chromatography. Wiley, New York, 1992.

Chapter 4

Characterization of lignin solubilised from two energy biomasses (*Miscanthus χ Giganteus*, and *Arundo donax*, L.) by derivatization followed by reductive cleavage (DFRC) and ^{31}P-NMR spectroscopy

Introduction

Biomass-based fuels and goods have attracted the attention of scientific community and people in the last decades, because of the environmental concerns about the usage of traditional fossil fuels. Plant biomass is composed mainly of cellulose, lignin and hemicellulose. Cellulose, the most abundant constituent of plant cell wall, is the main component extracted from biomass to obtain marketable products. After cellulose has been extracted, the remains are usually discarded or burnt (Cherubini, 2010). To avoid the waste of potentially useful compounds, lignin and hemicellulose have been thought to be used in the industry. In particular, lignin can be used to yield either simple organic compounds, such as toluene, 4-hydroxy-3-methoxybenzaldehyde (vanillin), phenol (Zakzeski et al., 2010), biocomposites (Mansur et al, 2005) or polyblends (Feldman, 2002). The ideal energy crop plants to be cultivated for this purpose should be perennial and should not need either a large amount of nutrient or big soil treatments. In addition, their production should be very consistent. Two of the plants meeting these requirements are miscanthus (*Miscanthus χ Giganteus*, Greef et Deuter) and giant reed (*Arundo donax*, L.) (Angelini et al., 2005; Heaton et al., 2004a; Heaton et al., 2004b; Lewandoski et al., 2000).

Besides the choice of initial biomass, another very important issue is the achievement of suitable extraction method to isolate purified lignin components. A large array of different methodologies has been applied to separate lignin from the plant biomasses (Zakzeski et al., 2010).

One of the most environmental friendly techniques is based upon the use of hydrogen peroxide (H_2O_2) at alkaline pH (Gould, 1984). At these conditions, the hydrogen peroxide reacts with lignocellulosics yielding low molecular weight, water-soluble phenols and hemicelluloses. Hemicellulose is then separated from lignin by lowering the pH down to 5.5 and by diluting the solution with ethanol, thus allowing the flocculation of hemicellulose. After the filtration of hemicellulose, lignin is recovered by evaporating the ethanol and by lowering the solution pH to 1.5-2.0 (Sun et al., 2000). The resulting lignin has lower molecular weight and larger amount of carbonyl and carboxyl functionalities respect to lignin residues commonly extracted with other techniques such as Kraft or sulfite methods (Holladay et al., 2007). The successful and effective utilization of lignin for, either, industrial applications or agronomical bio-technologies as humic-like plant growth promoter is based on its further degradation to monomeric structures (Doherty et al., 2011). Therefore after the isolation from plant biomasses, lignin has to be fully depolymerised and carefully characterised, to determine the suitability of physical-chemical properties for the utilization requirements.

The Derivatization Followed by Reductive Cleavage (DFRC) method is a useful procedure to combine both depolymerisation and analytical objectives, since it promotes a conservative splitting of lignin components thereby allowing a detailed molecular characterization of the final compounds by Gas Chromatography – Mass Spectrometry (GC-MS) analyses. The DFRC method is based on the break-down of typical β-O-4 lignin bonds by means of zinc powder and controlled processing conditions (up to 50°C), to produce relatively stable monomers and dimers and avoiding possibly secondary reactions (Lu & Ralph, 1998; Peng et al., 1998). The DRFC has been extensively implemented in lignin research and it has been successfully employed for understanding the biosynthetic pathways and to analyse amount and composition of different lignin structures. Notwithstanding the usefulness of DFRC method, the occurrence of not easily GC-detectable reaction products, such as carboxylates derivatives (e.g. benzoic acids, phenyl acetic acids, phenyl-

propanoic acids etc.) represent a limit for a comprehensive yield evaluations and may prevents the appraisal of specific lignin components (Guerra et al., 2008). To overcome this analytical hindrance and improve the performance of molecular characterization, the lignins extracted have been characterized with an additional technique based on the previous labelling of polar groups with ^{31}P marker (Figure 1), followed by the ^{31}P-NMR spectroscopy analysis (Tohmura & Argyropoulos, 2001). A scheme of the reactions the lignins have been subjected to in this study is reported in Figure 2. This technique has already been applied for both the analysis of lignin composition (Granata & Argyropoulos , 1995; Guerra et al., 2006; Sannigrahi et al., 2010) and to study the chemical mechanisms of specific lignin processing (Archipov et al., 1991; Crestini et al., 1999; Wörmeyer et al., 2011).

In this work the lignin residues previously extracted from both miscanthus and giant reed biomasses by using H_2O_2 at alkaline pH, were further depolymerised by DFRC method and extensively characterized by GC-MS and ^{31}P-NMR spectroscopy.

Materials and methods

Biomasses. Miscanthus was provided by Phytatec (UK) Ltd and was harvested on February. Instead, giant reed lignocellulosic material came from the experimental farm located in Bellizzi (Province of Salerno, Italy) owned by the University of Naples “Federico II” and it was cropped on January (Pirozzi et al., 2010). Miscanthus and giant reed will be referred to as MG and AD, respectively.

Chemical and reagents. Hydrogen peroxide (H_2O_2) was purchased from Merck KgaA. Ethanol was obtained from Carlo Erba Reagenti. Sodium hydroxide (pellet) was purchased from Clen Consult International SpA and it was reagent grade.

Alkaline oxidative hydrolysis. The alkaline oxidative extractive method was implemented as described elsewhere (Sun et al., 2000). Briefly, 5.0 g of samples were placed in a 150 mL of distilled water containing a 2 % H_2O_2 (v/v) solution. Then, the pH of the mixture was raised to 11.5 with a 4 M NaOH solution. The reaction was ended after 24 hours, by centrifuging the suspension (10,000 rpm x 10 min). The pH of supernatant (formed by lignin and hemicellulose) was lowered to 5.5 and 3 volumes of ethanol were added to make the hemicellulose flocculate. The mixture was separated by filtration. The ethanol present in the filtrate was, then, evaporated and the pH was lowered again to 1.5-2.0 to precipitate the lignin. The reaction mixture was centrifuged and the lignin recovered and freeze-dried. The oxidized lignin were analysed by DFRC and, then, by ^{31}P-NMR spectroscopy.

Derivatization followed by reductive cleavage (DFCR). The DFRC analytical method was nearly identical to that reported by Lu & Ralph (1997). A precise amount of lignin (50.0 mg) wes treated with 25 mL of a 1:9 (v/v) acetyl bromide solution for 3 h at 50°C under magnetic stirring. After this step, the solvent was evaporated under vacuum. Then, the sample was dissolved in a 5:4:1 dioxane:acetic acid:water solution and Zn powder (250 mg) was added to this solution. This step allows the β-O-4 aryl alkyl ether linkages to be broken down and double bond to be formed between Cα and Cβ. After 30 min at room temperature, the mixture was quantitatively transferred to a saturated NH_4Cl solution (7.5 mL) in a separating funnel using HPLC-grade dichloromethane (7.5 mL). The aqueous layer was extracted with dichloromethane (2 x 7.5 mL). The two extracts were dried with anhydrous Na_2SO_4 and then the solvent was evaporated to dryness under vacuum. The solution was then transferred to a 25 mL volumetric flask with dichloromethane and 5 mL of this solution were withdrawn for subsequent acetylation, which was carried out as follows: the solution formerly withdrawn was dried under N_2 stream and, then, pyridine (0.5 mL) and acetyl acetate (0.1 mL) were added. The reaction lasted 30 min at room temperature. After that, the

mixture was dried under N_2 stream. Finally, 10 μL of a 77.3 ppm hexadecane solution (in dichloromethane) and 990 μL of dichloromethane were added for GC-MS analysis.

Gas chromatography-Mass spectrometry. An aliquot of the previously prepared solution (2 μL) was injected (splitless mode) into a Perkin-Elmer Autosystem XL gas chromatograph equipped with a RTX-5MS WCOT capillary column, (Restek, 5% diphenyl-95% dimethylpolysiloxane, length 30 m, 0.25 mm ID, and 0.25 μm df) that was coupled, through a heated transfer line (250 °C), to a PE Turbomass-Gold quadrupole mass spectrometer. The injection port temperature was 270 °C, and the oven temperature was raised from 100 to 280 °C with a gradient of 8 °C/min. The internal standard used was hexadecane and the estimated correction factor was 6.96.

^{31}P-Nuclear Magnetic Resonance. Samples (12.0 mg) were placed in NMR tubes with 674 μL of $CDCl_3$,13 μL of N,N-dimethylformamide and 13 μL f pyridine. Two milligrams of triphenyl phosphate (Sigma-Aldrich) and 320 μg of chromium (III) acetylacetonate (Sigma-Aldrich) were used to calibrate the axis and as relaxing reagent, respectively. Finally, 100 μL of 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxa-phospholane (Sigma-Aldrich) were added to the solution. A 400 MHz Bruker Avance spectrometer, equipped with a 5 mm Bruker Inverse Broad Band (BBI) probe, working at ^{1}H frequency of 400.13 MHz, was employed to conduct ^{31}P liquid-state NMR measurements at a temperature of 298±1 K. ^{31}P-NMR spectra were acquired by adopting an inverse gated pulse sequence, including 80 μs length (15.6 dB power level) Waltz16 scheme in order to decouple phosphorous from proton nuclei. The experiments were acquired by selecting an initial 45° pulse length of 7.8 μs and a spectral width of 400 ppm (64,935.066 Hz), where the on-resonance frequency was set to 100 ppm. Each spectrum consisted in a 10 s thermal delay, 304 transients, 8 dummy scans and 65,536 time domain points. All spectra were baseline corrected and processed by both Bruker Topspin Software (v.2.1) and MestReC NMR Processing (v. 4.9.9.9). The Fourier Transform of FIDs (Free Induction Decays) was conducted by applying a 1.5 Hz

exponential multiplication as well as no zero-filling execution. ^{31}P frequency axis was calibrated by associating the value of -17.495 ppm to the TPP resonance.

Results and Discussion

Yield and composition of DFRC products. The physical chemical characteristics of the lignins extracted from miscanthus (MG) and giant reed (AD) by alkaline oxidative hydrolysis are reported in chapter 3. Both the lignins have shown to be extensively depolymerized and largely oxidized, due to the nature of the hydrolytic process (Kadla, 1999; Yamashita et al., 2010). Also, they are highly water-soluble, and their molecular weight is lower than 3,000 Da, as assessed by high performance size exclusion chromatography.

The GC-MS analysis of lignin components obtained by the DFRC of lignin extracts from MG and AD plants have identified more than 40 different monomers for each analysed biomass (Figure 3). The specific compounds, listed in Tables 1 and 2 for MG and AD plants respectively, are associated with current symbolism used for the identification of lignin basic structures (Figure 4): P, *para*-hydroxyphenyl; G, guaiacyl (3-methoxy, 4-hydroxyphenyl); and S, syringyl (3,5-dimethoxy, 4-hydroxyphenyl) (Vane et al., 2001; Spaccini and Piccolo 2007; Lu and Ralph, 1998). The various lignin derivatives released by DFRC from both biomasses (Tables 1 and 2) are typical constituents of lignocellulose fractions of herbaceous plants (Freudenberg & Neish, 1968). In fact, while the softwood is almost exclusively made up of guaiacyl sub-units and both syringyl and guaiacyl monomers constitute the hardwood, all three components form the building blocks of grass lignin, in different ratios depending on plant species (Goñi & Hedges, 1992).

The data in Table 3 reveal a significant difference in the cumulative yields of different lignin units. The amount of G for MG is more than the double than the one for AD, thus highlighting the differences between the two substrates from a biochemical point of view. The G-based units are the largest released monomers in both the studied biomasses, followed, with a progressive decreasing content, by S- and H-based molecules for AD (Tables 2 and 3), whereas both these classes are present in similar amounts in MG (Tables 1 and 3).The most abundant compound for both the substrates is the *cis*-guaiacyl alcohol which represents about the 32.2 mole percentage (312.6 μmol/g) and 38.6 mole percentage (193.5 μmol/g) of the aromatic compounds identified for MG and AD, respectively (Tables 1 and 2). Instead, the following representative compounds found in the chromatograms are the *trans*-guaiacyl alcohol for MG (95.0 μmol/g of lignin) and the *cis*-sinapyl alcohol for AD (139.0 μmol/g of lignin), while a lower content, for both plants, is shown by the *cis*- and *trans*- isomers of *p*-coumaryl alcohol, with a total concentration of 45.9 and 10.6 μmol/g for MG and AD in the order (Tables 1 and 2).

Although the guaiacyl-derivatives are considered the main components of lignified tissues in both MG and AD plants, followed by syringyl- and *para*-coumaryl-based molecules, large differences have been reported with respect to the relative amounts of the three lignin sub-units (Joseleau et al., 1976; Joseleau and Barnoud, 1976; Tai et al., 1987; Faix et al., 1989; Pascoal Neto et al., 1997; Seca et al., 2000; El Hage et al., 2009; Marques et al., 2010). Irrespective to the specific variety and cropping or natural growing conditions, these differences have also to be ascribed to the different lignin extraction methods. Our data, based on the alkaline oxidative delignification, indicate a prevalence of G-units for MG plant, with a G/S/P ratio equal to 6.3/1.4/1, while recent findings on lignin obtained by organic solvent extraction (El Hage et al., 2009) reported G-type compounds as the most prominent lignin units, with a very different G/S/P ratio (13/11/1). Moreover, the lignin residues obtained by Tai et al. (1987) and by Joselau et al., (1976) were isolated by giant reed after soda and Kraft pulping, respectively, with high temperature processes, while Pascoal Neto et al.

(1997) extracted lignin by using the permanganate oxidation method. These reaction conditions may promote the hydrolysis of ester bonds of P-based acid structures, thus partially explaining the discrepancy in the molecular composition of lignin. Furthermore, the differences could be due to a different period of harvesting. For instance, the S/G ratio for MG species may range from an average of 0.64 for the plants collected in autumn to an average of 0.77, for the winter harvesting (Le Ngoc Huyen et al., 2010). On the contrary, the S/G ratio for AD plants remains quite stable, whatever the crop cycle and harvesting period (Pascoal Neto et al., 1997). Finally, the G/S/P ratio obtained in the present work may be also related to the specific analytical steps of DFRC methods. The final acetylation of hydroxyl substituent is not able to react with the phenolic acids functional groups, deriving, from either the oxidative cleavage of lignin extracts or already present in the native lignin (Tai et al., 1987; Lygin et al., 2011), thus preventing an accurate appraisal of these components.

Some other aromatic molecules have been identified in GC-MS analyses of DFRC extracts (Tables 1 and 2). These compounds have been reported as constituents of the native lignin (Achyuthan et al., 2010) and have also been found in previous characterization of plant biomasses (Lu & Ralph, 1998; Ralph & Hatfield, 1991; Meier & Faix, 1992). For instance, keto-units arise from the cleavage of lignin dimers where the Cα is linked to an oxygen with a double bond (Lu & Ralph, 1998). The DFRC has been reported to be able to break down the 60% of these units (Lu & Ralph, 1997). Indeed, some brominated molecules have been identified. They arise from an incomplete hydrolysis of the compounds produced during the first step of DFRC when reacting with Zn (Lu & Ralph, 1998). Instead, hydroquinone may be formed during the reaction of lignin monomers with H_2O_2, via a Dakin-like reaction. Kadla et al. (1997) and Gellestedt & Agnemo (1980) have studied the reactions of H_2O_2 with lignin monomers or lignin model compounds at alkaline pH. They found that the hydroperoxide anion can react with both benzyl alcohols and alkyl

phenyl ketones, thus producing hydroquinones and, contemporarily, causing the loss of small polar organic molecules, such as simple aldehydes or carboxylic acids.

As seen in Tab. 3, also some carbohydrates and unidentified compounds have been found in the samples. Their concentration was calculated as percentage on the total area. Carbohydrates may arise from an inefficient separation of lignin from plant cell sugars and their occurrence has already been reported in the previous chapter (chapter 3). This fraction is mainly composed of xylose, in both pyranosyl and furanosyl isomers, for both the biomasses studied. This finding is confirmed by many past studies where the hemicellulose composition of miscanthus and giant reed were studied (Joseleau & Barnoud, 1975; de Vrije et al., 2002; Peng et al., 2012). Assigning the mass spectrum of an acetylated carbohydrate can be nontrivial, because the very low intensity of the signals of the molecular ion and of the first fragment. Luckily, the xylose mass spectrum can easily been found in the literature (Sárossy et al., 2012) so that the assignment of the mass spectra of the carbohydrates found to xylose has been easily carried out. The amount of carbohydrates is very low for all the biomasses studied and accounts for the 2.7 and the 3.2% of the total chromatographic area for MG and AD, respectively. Finally, the class “Others” contains all the compounds that have not been identified. They account for the 9.3% and 0.5% of the total area of the chromatograms for MG and AD, respectively.

^{31}P NMR spectrometry. ^{31}P-NMR spectroscopy has been shown to be a very useful tool to reveal the structure of lignin deeply (Crestini et al., 1999; Tohmura & Argyropoulos, 2001; Hattalli et al., 2002; Guerra et al., 2006; Sanniraghi et al., 2010). The existence of acidic groups is confirmed by ^{31}P-NMR spectrometry (Figure 5). Carboxylic acids resonate around between 135.6 and 133.7 ppm, whereas peaks in the range of 143.7–142.2 ppm are associated to S phenolic units. Signals in the range of 142.8–141.7 and 141.7–140.2 ppm are due to the resonance of condensed phenolic units (4-O-5’ type and 5-5’ type, respectively). Also, G units are associated to the

resonances between 140.2 and 138.4 ppm, whilst H resonances are around 138.6–136.9 ppm (Argyropoulos et al., 1993; Argyropoulos, 1995). Finally, the peaks around 146.3-150.8 may be related to the resonance of aliphatic OH (Argyropoulos, 2010). The signal around 141.7-140.2 ppm confirms the presence of condensed lignin rings, indicated by the peak at 1328 cm^{-1} in the infrared spectra reported in the previous chapter. Interestingly, the S/G ratio obtained by ^{31}P-NMR spectroscopy is 1.5 for MG and 2.3 for AD, whilst the S/G ratio calculated form chromatographic data is 0.2 and 0.7, for MG and AD, respectively. This much higher amount of syringyl units present in the NMR spectra than in the chromatograms is due, according to us, to the presence of some carboxyl functional groups, which cannot be observed in GC, because they cannot be acetylated. This hypothesis is confirmed by the peak at 135.2 ppm, attributed to the syringic acid (Spyros & Dais, 2000).

Conclusions

In this paper, the lignin extracted from miscanthus and giant reed was characterized by gas chromatography and liquid-state ^{31}P NMR spectroscopy. The data obtained show that G-type molecules are the most represented for both the substrates and that DFRC is confirmed to be an important tool to study the lignin heterogeneous structure. The gas chromatographic analysis confirms the presence of carbohydrates, even if in very low amounts, thus showing that the extractive method chosen gives a high purity lignin (purity > 95% of the total chromatographic area). Lastly, ^{31}P NMR spectroscopy has revealed the occurrence of some phenolic acids, making it clear that not all compounds coming from the alkaline extractive method can be analyzed by GC using the DFRC protocol. This indicates that DFRC is not a suitable method to study those lignins where acidic functionalities are present, because they cannot be derivatized by following the

original DFRC protocol. This study also shows that the usage of ^{31}P-NMR spectroscopy can overcome this limitation, allowing a complete qualitative analysis of the lignin in exam.

Table 1. Yields of the different lignin-derived molecules as by GC-MS chromatograms of DFRC extracts of lignin separated from MG.

RT (min)	Attribution	Concentration (μmol/g)
6.0	G-CH_3[a]	51.7
7.2	Hydroquinone	3.4
7.4	P-$CH_2CH_2CH_3$[b]	79.1
7.5	P-CH_2COCH_3	3.8
8.2	G-$CH_2CH{=}CH_2$	2.8
8.3	G-CHO	25.7
8.7	P-CH_2OH	2.1
8.7	S-CH_3[c]	3.0
9.3	HEXADECANE	-
9.3	G-$CH_2CH{=}CH_2$	10.2
9.6	G-$COCH_3$	32.2
10.1	G-C≡CCH_3	3.3
10.5	G-CH_2COCH_3	48.2
10.7	*trans*-S-CH=$CHCH_3$	1.2
10.7	*cis*-S-CH=$CHCH_3$	1.2
10.9	G-CH_2OH	25.3
10.9	G-CO-CH_2CH_3	4.5
11.2	S-CH_2-CH=CH_2	4.2
11.2	Carbohydrate[d]	-
11.5	Carbohydrate	-
11.7	*trans*-P-CH=CHCH2OH	5.9

11.8	*trans*-G-cPr-OH[e]	6.8
11.8	S-cPr	6.6
11.9	Carbohydrate	-
12.0	P-$CH_2CH_2CH_2OH$	2.5
12.0	Carbohydrate	-
12.1	Other[d]	-
12.6	*cis*-P-CH=CH-CH_2OH	40.0
12.7	S-CH_2CO-CH_3	13.5
12.8	S-COCH=CH_2	3.1
12.8	Other	-
13.0	Other	-
13.2	G-$CH_2CH_2CH_2OH$	8.4
13.4	Other	-
13.5	Other	-
13.6	*trans*-G-CH=CH-CH_2OH	95.0
13.7	*trans*-S-cPrOH	2.1
14.0	Other	-
14.1	S-CHOH-CH=CH_2	5.6
14.2	Others	-
14.4	Other	-
14.8	*cis*-G-CH=CH-CH_2OH	312.6
14.9	*trans*-G'-CH=$CHCH_2OH$[f]	7.2
15.0	*cis*-G-cPr-OH	9.4
15.1	S-CHOH-CO-CH_3	0.7
15.5	*trans*-S-CH=CH-CH_2OH	24.5

15.6	G-CHOH-CH_2-CH_2OH	9.5
16.1	*cis*-G'-CH=$CHCH_2OH$	23
16.5	Other	-
16.7	*cis*-S-CH=$CHCH_2OH$	63.3
17.3	Other	-
17.4	G-CHOHCHOH-CH_2OH	9.9
17.7	G-CHOHCHOH-CH_2OH	1.5
17.9	G''-CH=$CHCH_2OH$[g]	14.6
18.3	S-CHOHCHOH-CH_2OH	1.9
18.6	S-CHOHCHOH-CH_2OH	1.4

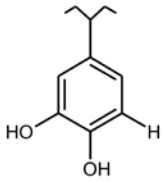
a. G: guaiacyl-type units. The concentration is calculated based on the guaiacyl alcohol (180 Da).

b. P: *para*-coumaryl units. The concentration is calculated based on the *para*-coumaryl alcohol (150 Da).

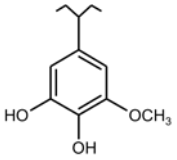
c. S: syringyl-type units. The concentration is calculated based on the sinapyl alcohol (210 Da).

d. Individual yield not reported for "carbohydrate" and "other" classes.

e. cPr: cyclopropyl.

f. G': 

concentration calculated on its molecular weight: 166 Da.

g. G'': 

concentration calculated on its molecular weight: 196 Da.

Table 2. Yields of the different lignin-derived molecules as by GC-MS chromatograms of DFRC extracts of lignin separated from AD.

RT (min)	Attribution	Concentration (μmol/g of lignin)
6.03	G-CH_3[a]	2.4
7.20	Hydroquinone	0.7
7.39	P-$CH_2CH_2CH_3$[b]	2.1
7.52	P-CH_2COCH_3	7.0
7.78	P-$CH{=}CH_2$	3.8
8.18	G-$CH_2CH{=}CH_2$	1.4
8.31	G-CHO	2.7
8.67	P-CH_2OH	0.3
8.72	S-CH_3[c]	3.6
9.34	HEXADECANE	-
9.37	G-$CH_2CH{=}CH_2$	1.2
9.46	G-$CH{=}CHCH_3$	3.5
9.57	G-OH	0.2
9.64	G-$COCH_3$	1.8
9.79	G-$CH{=}CH_2$	0.1
10.06	G-C≡CCH_3	2.4
10.46	G-CH_2COCH_3	14.4
10.55	G-CH_2CH_2CHO	0.6
10.68	*trans*-S-$CH{=}CHCH_3$	1.7
10.74	*cis*-S-$CH{=}CHCH_3$	0.7
10.87	G-CH_2OH	8.7

10.91	G-CO-CH_2CH_3	3.1
11.17	S-CH_2-CH=CH_2	5.0
11.24	Carbohydrate[d]	-
11.50	Carbohydrate	-
11.68	*trans*-P-CH=CH-CH_2OH	1.2
11.74	*trans*-G-cPr-OH[e]	4.5
11.82	S-cPr	2.4
11.87	Carbohydrate	-
11.97	P-$CH_2CH_2CH_2$OH	3.2
12.00	Carbohydrate	-
12.56	*cis*-P-CH=CH-CH_2OH	9.4
12.68	S-CH_2CO-CH_3	4.2
12.78	S-COCH=CH_2	0.6
12.85	Other[d]	-
13.09	S-CH_2OH	5.1
13.17	G-$CH_2CH_2CH_2$OH	4.5
13.52	*trans*-G-CH=CH-CH_2OH	28.7
13.65	*trans*-S-cPrOH	3.8
14.10	S-CHOH-CH=CH_2	1.5
14.76	*cis*-G-CH=CH-CH_2OH	193.5
14.88	*trans*-G′-CH=CHCH_2OH[f]	1.3
14.97	*cis*-G-cPr-OH	3.6
15.05	S-CHOH-CO-CH_3	1.2
15.45	*trans*-S-CH=CH-CH_2OH	4.4
15.56	*cis*-S-cPrOH	0.8

15.59	G-CHOH-CH_2-CH_2OH	1.8
15.73	G-CHOHCH_2CH_2Br	1.3
16.05	*cis*-G'-CH=CHCH_2OH	2.3
16.70	*cis*-S-CH=CHCH_2OH	139.0
17.08	G-CH=CHOH-CHO	2.9
17.15	G-CHOH-CHOH-CH_2OH	0.6
17.28	Other	-
17.38	G-CHOH-CHOH-CH_2OH	1.7
17.50	Other	-
17.85	G''-CH=CHCH_2OH[g]	3.3
18.35	S-CHOH-CHOH-CH_2OH	1.3
18.59	S-CHOH-CHOH-CH_2OH	1.9

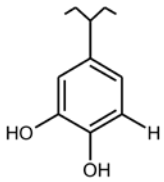
a. G: guaiacyl-type units. Their concentration was calculated based on the guaiacyl alcohol (180 Da).

b. P: *para*-coumaryl units. Their concentration was calculated based on the *para*-coumaryld alcohol (150 Da).

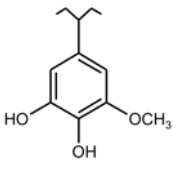
c. S: syringyl-type units. Their concentration was calculated based on the sinapyl alcohol (210 Da).

d. Individual yield not reported for "carbohydrate" and "other" classes.

e. cPr: cyclopropyl.

f. G': 

concentration calculated on its molecular weight: 166 Da.

g. G'': 

concentration calculated on its molecular weight: 196 Da.

Table 3. Yields of DFRC extracts as calculated from GC-MS chromatograms of the two lignins separated from lignocellulose biomass.

Lignin fragments[a]						
μmol/g of lignin					**Percentage of the total chromatographic area**	
Lignin	**G**[b]	**S**[c]	**P**[d]	**Hy**	**C**	**O**
MG	656	132.3	133.5	3.4	2.7	9.3
AD	285.8	177.2	27	0.7	3.2	0.5

a. G: guaiacyl-type units; S: syringyl-type units; P: *para*-coumaryl units; Hy: hydroquinone; C: carbohydrates; O: others and unknown.

b. concentration calculated based on the guaiacyl alcohol (180 Da).

c. concentration calculated based on the sinapyl alcohol (210 Da).

d. concentration calculated based on the para-coumaryl alcohol (150 Da).

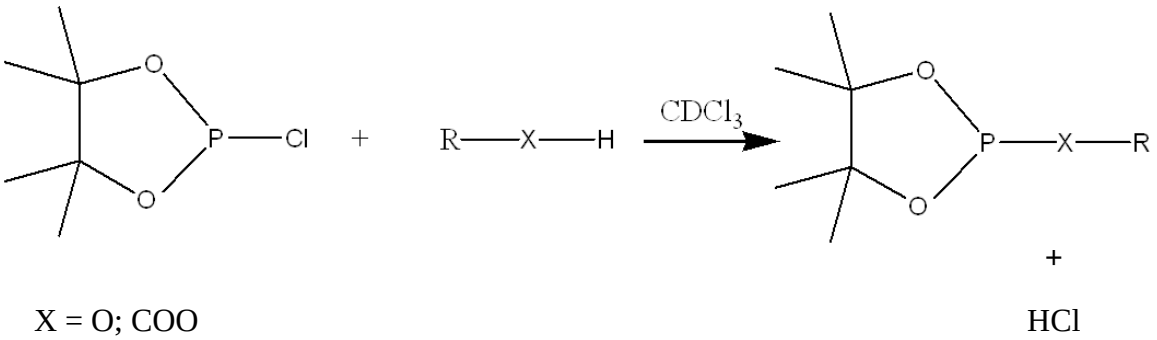


Figure 1. Derivatization reaction of hydrixyl, phenolic and carboxyl groups with phosphytilating agent (2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxa-phospholane).

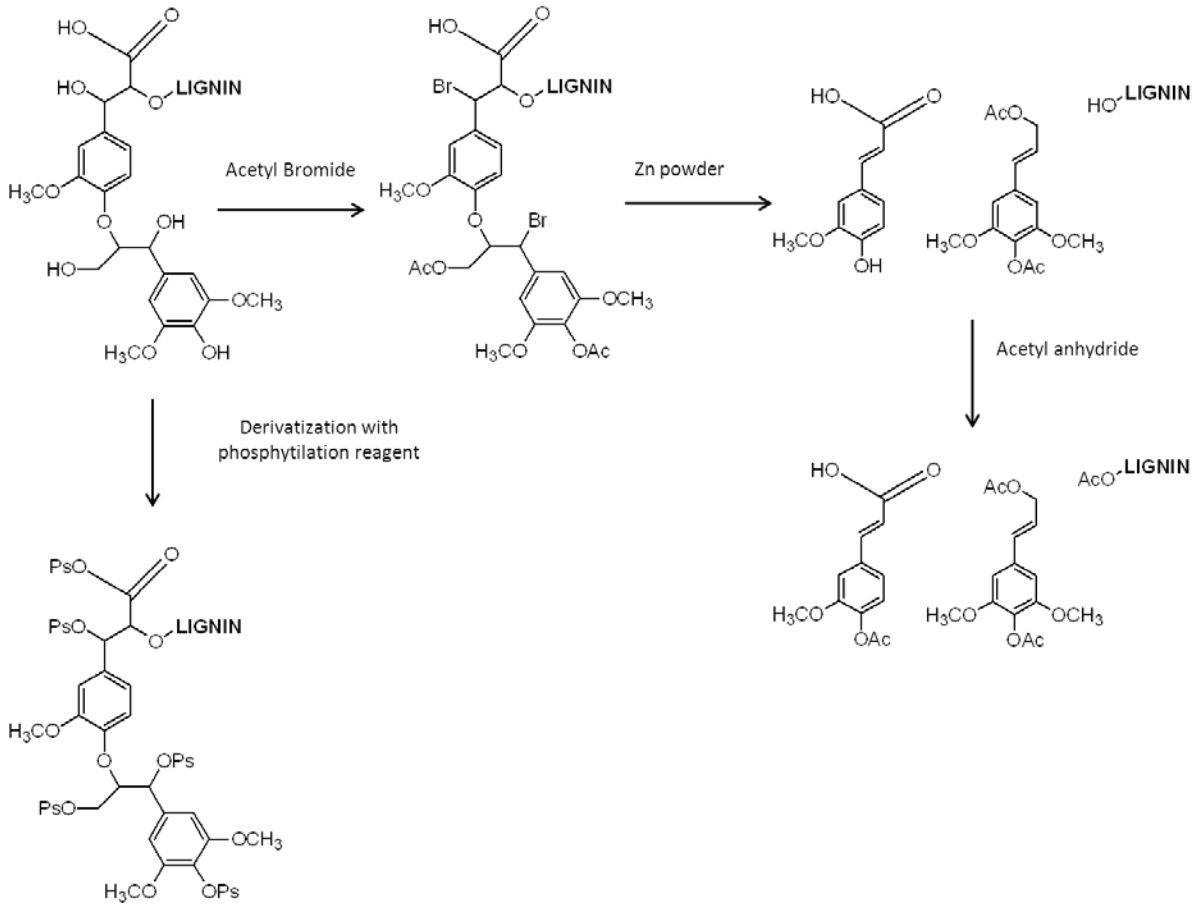


Figure 2. Reactions involved in the DFRC analytical method and derivatization with 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxa-phospholane (Ps) for ^{31}P-NMR spectroscopy study of a dlignin dimer containing a carboxyl functionality.

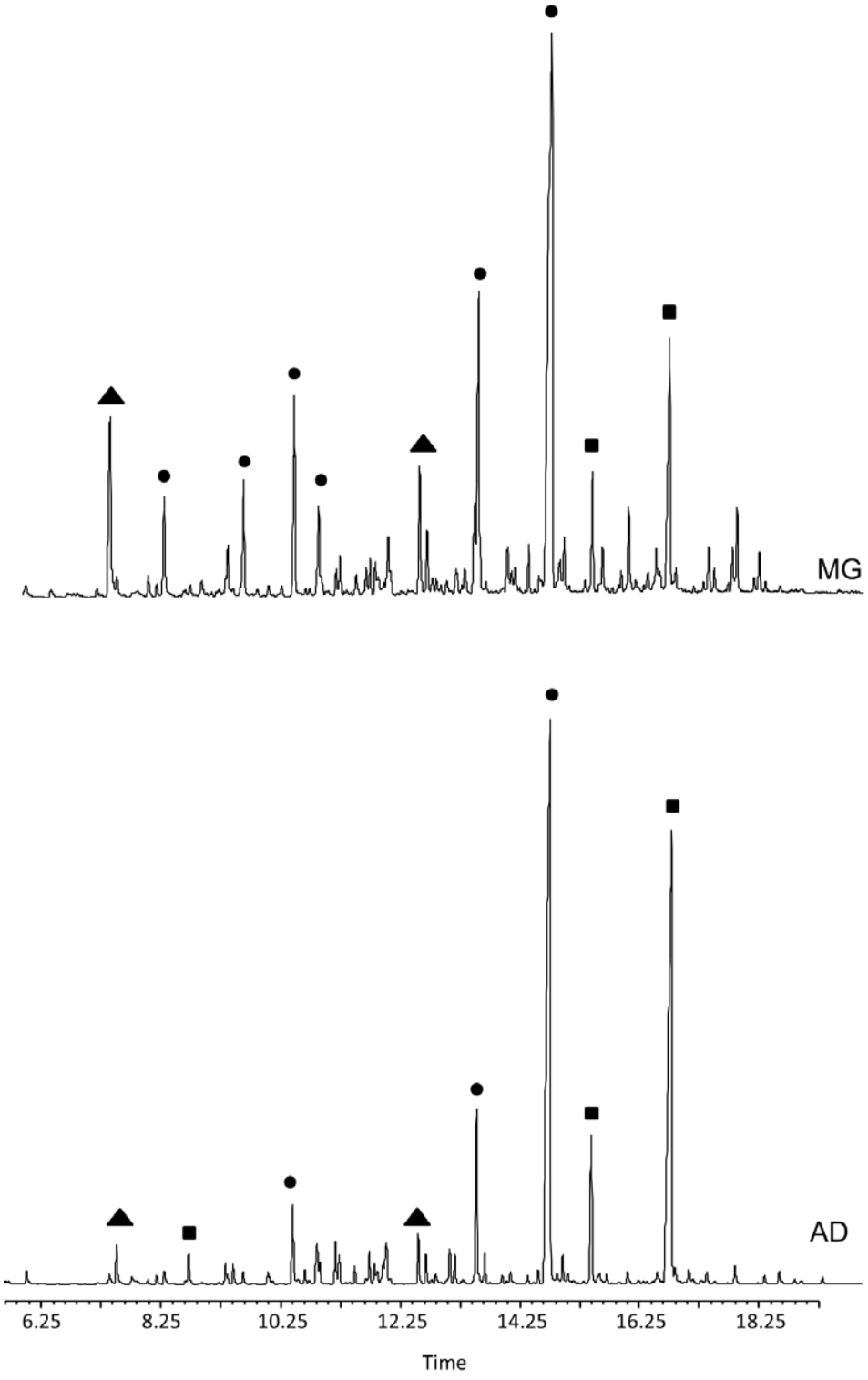


Figure 3. Gas chromatograms after DFRC procedure for lignin extracted by miscantus (MG) and giant reed (AD) biomass. ▲: *para*-coumaryl-based units; ●: guaiacyl-based units; ■: syringyl-based units.

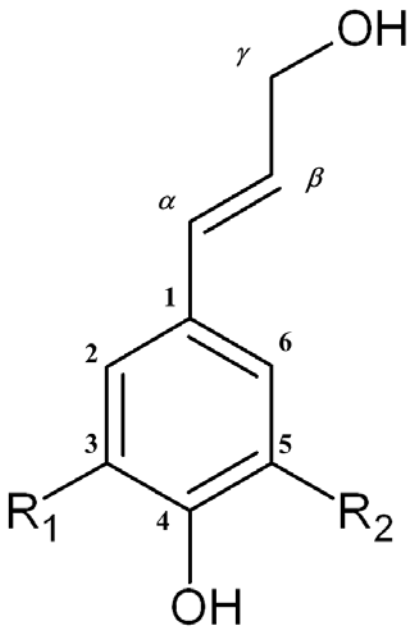


Figure 4. Lignin monomers. *Para*-coumaryl unit (P): R_1 = H; R_2=H. Guaiacyl unit (G): R_1=H; R_2=OCH_3. Sinapyl unit (S): R_1 and R_2=OCH_3. Numbers indicate carbon atoms.

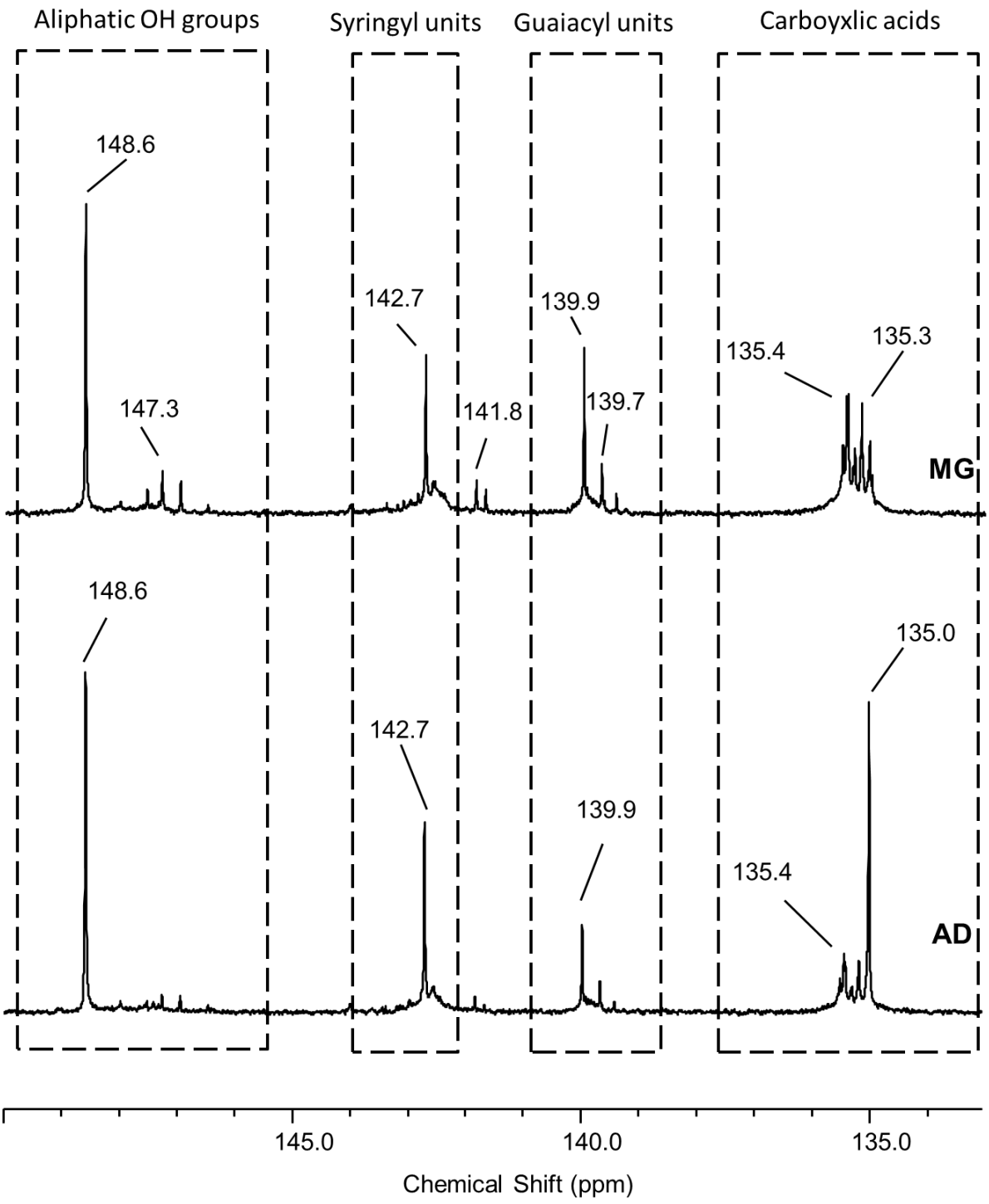


Figure 5. ^{31}P-NMR spectra for lignins extracted by miscanthus (MG) and giant reed (AD) biomass.

References

Angelini, L G; Ceccarini, L; Bonsri, E (2005) Biomass yield and energy balance of giant reed (*Arundo donax*, L.) cropped in central Italy as related to different management practices European Journal of Agronomy 22, 375–389.

Archipov, Y; Argyropoulos, D S; Bolker, H I; Heitner, C (1991) ^{31}P NMR spectroscopy in wood chemistry. Part I. Lignin model compounds, Journal of Wood Chemistry and Technology, 11, 137–157.

Argyropoulos, D S (1995) ^{31}P NMR in Wood Chemistry, A Review of Recent Progress. Research on Chemical Intermediates, 21, 373-395.

Argyropoulos, D S; Bolker, H I; Heitner, C; Archipov, Y(1993) ^{31}P NMR Spectroscopy in Wood Chemistry. Part V, Qualitative Analysis of Lignin Functional Groups. Journal of Wood Chemistry and Technology, 13, 187-212.

Argyropoulos, D S; Heteronuclear NMR Spectroscopy of Lignins. In: Lignin and Lignans: Advances in Chemistry; Heitner, C; Dimmel, D R; Schmidt, J A, Eds; Taylor and Francis Group, LLC- Boca Raton, 2010 , pp 245-265.

Cherubini, F (2010) The biorefinery concept: Using biomass instead of oil for producing energy and chemicals. Energy Conversion and Management 51, 1412–1421.

Crestini, C & Argyropoulos, D S (1997) Structural Analysis of Wheat Straw Lignin by Quantitative ^{31}P and 2D NMR Spectroscopy. The Occurrence of Ester Bonds and β-O-4 Substructures, Journal of Agricultural and Food Chemistry, 45, 1212 − 1219.

Crestini, C Saladino, R; Tagliatesta, P; Boschi T (1999) Biomimetic Degradation of Lignin and Lignin Model Compounds by Synthetic Anionic and Cationic Water Soluble Manganese and Iron Porphyrins. Bioorganic & Medicinal Chemistry, 7, 1897–1905.

de Vrije, T; de Haas, G G; Tan, G B; Keijsers, E R P; Claassen, P A AM (2002) Pretreatment of Miscanthus for hydrogen production by *Thermotoga elfi*. International Journal of Hydrogen Energy 27, 1381–1390.

Doherty, W O S, Mousavioun, P, Fellows C M (2011). Value-adding to cellulosic ethanol: Lignin polymers. Industrial Crops and Products 33, 259–276.

El Hage, R; Brosse, N; Chrusciel, L; Sanchez. C; Sannigrahi, P; Ragauskas, A (2009) Characterization of milled wood lignin and ethanol organosolv lignin from *miscanthus*. Polymer Degradation and Stability 94, 1632-1638.

Faix, O; Meier, D; Beinhoff, O (1989) Analysis of lignocelluloses and lignins from *Arundo donax* and *Miscanthus sinensis* Anderss and hydroliquefaction of Miscanthus. Biomass 18, 109.

Feldman, D, 2002. Lignin and its polyblends—a review. In: Hu, T.Q. (Ed.), Chemical Modification, Properties, and Usage of Lignin. Springer, New York, pp. 81–100.

Freudenberg, K; Neish, A C, eds. Constitution and Biosynthesis of Lignin, Molecular-Biology Biochemistry and Biophysics, Vol. 2. Springer-Verlag; Berlin, 1968; 129 pp.

Gellestedt, G & Agnemo, R (1980) The Reactions of Lignin with Alkaline H2O2. Part III. The Oxidation of Conjugated Carbonyl Structures. Acta Chemica Scandinavia B, 34, 275-280.

Gould, J M (1984). Alkaline peroxide delignification of agricultural residues to enhance enzymatic saccharification. Biotechnology and Bioengineering, 26, 46-52.

Granata, A & Argyropoulos, D S (1995) 2-Chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane, a reagent for the accurate determination of the uncondensed and condensed phenolic moieties in lignins. Journal of Agricultural and Food Chemistry 43, 1538–1544.

Guerra, A; Filipponen, I; Lucia, L A; Argyropoulos, D (2006) Comparative Evaluation of Three Lignin Isolation Protocols for Various Wood Species. Journal of Agricultural and Food Chemistry, 54, 9696−9705.

Guerra, A; Filpponen, I;Lucia, LA; Saquing, C; Baumberger, S; Argyropoulos, D S (2006) Toward a Better Understanding of the Lignin Isolation Process from Wood. Journal of Agricultural and Food Chemistry, 54, 5939-5947.

Guerra, A; Norambuena, M; Freer, J; Argyropoulos, D S (2008) Determination of Arylglycerol -β-Aryl Ether Linkages in Enzymatic Mild Acidolysis Lignins (EMAL): Comparison of DFRC/ 31 P NMR with Thioacidolysis. Journal of Natural Products 2008, 71, 836–841.

Hattalli, S; Benaboura, A; Ham-Pichavant, F; Nourmamode, A; Castellan, A (2002) Adding value to Alfa grass (Stipa tenacissima L.) soda lignin as phenolic resins1. Lignin characterization. Polymer Degradation and Stability, 75, 259–264.

Heaton E, Voigt T, Long S P (2004a) A quantitative review comparing the yields of two candidate C4 perennial biomass crops in relation to nitrogen, temperature and water. Biomass and Bioenergy, 27, 21–30.

Heaton, E A; Clifton-Brown, J; Voigt, T; Jones M B; Long S P (2004b) Miscanthus for renewable energy generation: European Union experience and projections for Illinois. Mitigation Adapt. Strategies Global Change, 9, 433–451.

Holladay, J E; Bozell, J J; White, J F; Johnson, D (2007). Top Value-Added Chemicals from Biomass Volume II—Results of Screening for Potential Candidates from Biorefinery Lignin. Pacific Northwest National Laboratory, U. S. Dempartment of Energy.

Joseleau, J P & Barnoud, F (1976) Cell wall carbohydrates and structural studies of xylan in relation to growth in the *Arundo donax*. Applied Polymer Symposia, 28, 983-992.

Joseleau, J P & Barnoud, F; (1975) Hemicellulose of *Arundo donax* at different stages of maturity. Phytochemistry, 14, 71-75.

Joseleau, J P; Miksche, G E; Yasuda, S (1976) Structural variation of *Arundo donax* lignin in relation to growth. Holzforschung 31, 19-20.

Kadla, J; Chang, H; Jameel, H (1997) The Reactions of Lignin with Hydrogen Peroxide at High Temperature. Part I. The Oxidation of Lignin Model Compounds. Holzforsching, 51, 428-434.

Le Ngoc Huyen, T; Rémond, C; Dheilly, R M; Chabbert, B (2010) Effect of harvesting date on the composition and saccharification of *Miscanthus χ Giganteus*. Bioresource Technology, 101, 8224–8231.

Lewandowski, I; Clifton-Brown, J C; Scurlock, J M O; Huisman W (2000) Miscanthus: European experience with a novel energy crop. Biomass and Bioenergy, 19, 209–27.

Lu, F; Ralph, J (1997) Derivatization Followed by Reductive Cleavage (DFRC method), a New Method for Lignin Analysis: Protocol for Analysis of DFRC monomers. Journal of Agricultural and Food Chemistry, 45, 2590 - 2592.

Lu, F; Ralph, J (1998) The DFRC Method for Lignin Analysis. 2. Monomers from Isolated Lignins. Journal of Agricultural and Food Chemistry, 46, 547-552.

Lygin, Upton, J; Dohleman, F G;Juvik, J; Zabotina, O A; Widholm, J M; Lozovaya, V V (2011) Composition of cell wall phenolics and polysaccharides of the potential bioenergy crop – Miscanthus. Global Change Biology Bioenergy 3, 333-45.

Mansur, H S; Mansur, A A P; Bicallho, S M C M (2005) Lignin-Hydroxyapatite/Tricalcium Phosphate Biocomposites: SEM/EDX and FTIR Characterization. Key Engineering Materials, 284-286, 745-748.

Marques, G; Rencoret, J; Gutiérrez, A; del Río, J C (2010) Evaluation of the Chemical Composition of Different Non-Woody Plant Fibers Used for Pulp and Paper Manufacturing. The Open Agriculture Journal, 4, 93-101.

Meier, D & Faix, O; Pyrolysis-Gas-Chromatography-Mass-Spectrometry. In Methods in lignin chemistry; Lin, S Y, Dence, C W, Eds.; Springer-Verlag: Berlin, 1992; pp 177-199.

Pascoal Neto, C; Seca, A; Nunes, A M; Coimbra, M A; Domingues, F; Evtuguin, D; Silvestre, A; Cavaleiro, J A S (1997) Variations in chemical composition and structure of macromolecular

components in different morphological regions and maturity stages of *Arundo donax*. Industrial Crops and Products, Volume 6, 51–58.

Peng, F; Bian, J; Peng, P; Xiao, H; Ren,, J; Xu, F, Sun, R (2012) Separation and Characterization of Acetyl and Non-Acetyl Hemicelluloses of *Arundo donax* by Ammonium Sulfate Precipitation. Journal of Agricultural and Food Chemistry, 4039-4047.

Peng, J; Lu, F; Ralph, J (1998) The DFRC Method for Lignin Analysis. 4. Lignin Dimers Isolated from DFRC-Degraded Loblolly Pine Wood. Journal of Agricultural and Food Chemistry, 46, 553 – 560.

Pirozzi D, Yousuf A, Fagnano M, Mori M, Fiorentino N, Impagliazzo A (2010) Lipids production by yeast grown on giant reed biomass. Journal of Biotechnology , 150, S1, 167-168.

Ralph, J & Hatfield, R D (1991) Pyrolysis-GC-MS Characterization of Forage Materials. Journal of Agricultural and Food Chemistry, 39, 1426-1437.

Sannigrahi, P; Ragauskas, A J; Miller, S J (2010) Lignin Structural Modifications Resulting from Ethanol Organosolv Treatment of Loblolly Pine. Energy Fuels, 24, 683 – 689.

Sárossy Z, Plackett D, Egsgaard H(2012) Carbohydrate analysis of hemicelluloses by gas chromatography-mass spectrometry of acetylated methyl glycosides. Analytical and Bioanalytical Chemistry, 403, 1923-1930.

Seca, A M L; Cavaleiro, J A S; Domingues, F M J; Silvestre, A J D; Evtuguin, D; Pascoal Neto, C (2000) Structural Characterization of the Lignin from the Nodes and Internodes of *Arundo donax* Reed. Journal of Agricultural and Food Chemistry, 48, 817 – 8247.

Spyros, A & Dais, P (2000) Application of 31P NMR Spectroscopy in Food Analysis. 1. Quantitative Determination of the Mono- and Diglyceride Composition of Olive Oils. Journal of Agricultural and Food Chemistry, 48, 802-805.

Sun, R, Tomkinson, J, Wang, S; Zhu, W (2000). Characterization of lignins from wheat straw by alkaline peroxide treatment. Polymer Degradation and Stability 67, 101-109.

Tai, D; Cho, W; Ji, W (1987) Studies of *Arundo donax* lignins. Proceedings of the 4th ISWPC, Vol. II, April 1987, Paris, pp. 13-17.

Tohmura, S & Argyropoulos, D S (2001) Determination of Arylglycerol-*β*-aryl Ethers and Other Linkages in Lignins Using DFRC/^{31}P NMR. Journal of Food Chemistry, 49, 536 – 542.

Wörmeyer, K; Ingram, T; Saake, B; Brunner, G; Smirnova, I (2011) Comparison of different pretreatment methods for lignocellulosic materials. Part II: Influence of pretreatment on the properties of rye straw lignin. Bioresource Technology 102, 4157–4164.

Zakzeski, J; Bruijnincx, P C A; Jongerius, A L; Weckhuysen, B M (2010) The Catalytic Valorization of Lignin for the Production of Renewable Chemicals. Chemical Reviews, 110, 3552–3599.

Chapter 5

Bioactivity of soluble Lignin separated from biomass for energy: effect on germination and early growth of *Zea mays*, L.

Introduction

In the last decades, the attention of the scientific community has been focussed on the production of fuels to substitute the petroleum-based ones. An environmental friendly way to achieve this purpose is the transformation of cellulose into ethanol thus using it as biofuel. The ideal plants to be grown for the production of biofuels and bio-based chemicals should be perennial and should need neither a large amount of nutrient nor big soil treatments. In addition, their production should be very consistent. Two of the plants showing these requirements are miscanthus (*Miscanthus χ Giganteus*, Greef et Deuter) and giant reed (*Arundo donax*, L.), (Angelini et al., 2005; Heaton et al., 2004a; Heaton et al., 2004b; Lewandoski et al., 2000).

Various methods can be employed to separate high purity cellulose from the rest of the plant cell wall (Sun & Cheng, 2002). Once cellulose has been extracted, some residues remain, which are usually discarded or burnt (Cherubini, 2010). These wastes are mainly composed of hemicellulose and lignin, which are still potentially useful for the production of various chemicals. For instance, hemicellulose can be used for the production of feed additives (Smiricky-Tjardes, 2003) and stimulators of the intestinal mineral absorption (Oku & Sadaku, 2002). Lignin, instead, can be used to yield either simple organic compounds, such as toluene, 4-hydroxy-3-methoxybenzaldehyde (vanillin), phenol (Zakzeski et al., 2010), or biocomposites, (Mansur et al., 2005).

Lignin could also be used as plant growth promoter, as humic-like substances (HULIS). Humic substances (HS) are commonly described as a complex of heterogeneous, natural organic

compounds, originated from the biochemical decay of plant and animal biomasses through microbial metabolism (Stevenson, 1994). According to Piccolo (2001), HS should be considered as a supramolecular association of low-molecular weight molecules. The HS chemical characteristics have been thoroughly studied by means of various analytical and spectroscopic techniques (Spaccini et al., 1998; Piccolo, 2001; Simpson, 2002; Lopez-Capel et al., 2005), highlighting that a considerable amount of aromatic, lignin-derived compounds is present in native soil humus (Nardi et al., 2007; Puglisi et al., 2009; Canellas et al., 2010). This has been related to the fact that lignin is one of the precursor of HS, together with other biomass-derived molecules. Both HS and HULIS affect plant growth and physiology in different ways, mainly by acting as hormone-like substances. The physiological effects of HS are very well known and have already been reported over one century ago (Robertson et al., 1907). Vaughan and MacDonald (1976) showed that HS can stimulate the development of an uptake capacity for Na^{+}, Ba^{2+} in beetroot disks. HS can also increase NO_3^{-} uptake (Vaughan et al., 1985), due to their gibberellin (GA)-like activity (Nardi et al., 2000). Moreover, HS positively affect photosynthesis (Sladky et al., 1959; Ferretti et al., 1991; Merlo et al., 1191) and respiration in higher plants (Vaughan and Malcom, 1985), by increasing, for example, the activity of the peroxidase (Muscolo et al., 1993). Canellas et al. (2008) and Busato et al. (2010) found that HS stimulated the elongation and proliferation of secondary maize roots. Instead, Dobbs et al., (2007) showed that HS influence the lateral root development in *Arabidopsis thaliana*, L. Furthermore, Canellas et al. (2002; 2009) proved that HS can increase the activity of the plasma membrane ATPase on maize seedlings, thus promoting the formation of lateral roots. HS have also a beneficial effect on the growth of whole plants. Eyheraguibel et al. (2008) reported an increase of both the total dry biomass of the plants employed and a higher amount of mineral elements adsorbed for treated plants than control ones after a two month experiment. Root application of HS may enhance the shoot growth in cucumber, by translocating several cytokinins and polyamines from the root to the aerial part, following to the nitrate change in the root:shoot

ratio (Mora et al., 2010). Indeed, the action of HS has been related to a positive increase of the plant yields. In a field experiment, Verlinden et al. (2009) found that HS enhance plant uptake of nitrogen, phosphorous, calcium and magnesium and induce an increase of crop production. Tufencki et al. (2006) reported a positive impact of HS application on the plant growth and nutrient contents of lettuce plants. Also, Atiyeh et al. (2002) proved that HS greatly enhance the growth of tomato and cucumber seedlings, till a concentration of 500 mg kg^{-1}, then the development of plants was significantly decreased. In a recent paper, Silva-Matos et al. (2012) showed that the foliar application of HS both promoted the growth of root and shoot and induced an increase in the leaf chlorophyll content of net-house grown watermelon seedlings. Moreover, HS have been noted to protect plants from either biotic or abiotic stresses. Kesba & El-Betagi (2012) reported a high resistance of grape toward nematode infections, while Loffredo et al. (2008) showed a positive effect of HS in inhibiting the growth of *Sclerotinia sclerotiorum* mycelium and contemporarily an increase of the growth of one of its antagonists, *Trichoderma viride*. García et al. (2012) showed that HS increase the tolerance of rice toward water stress.

In order to turn the plant lignin into HULIS for the application as plant growth promoter, it is essential to enhance the solubilization properties and simultaneously reduce the molecular weight of lignin derivatives, due to the supramolecular nature of HULIS and HS (Piccolo, 2001). Therefore, the choice of extraction and purification methods plays a key role in determining these physical-chemical characteristics.

In the present study the isolation of lignin components from miscanthus and giant reed biomasses has been performed with the use of hydrogen peroxide (H_2O_2) at alkaline pH (Gould, 1984). The oxidative alkaline conditions allow the lignocellulose biomass to be thoroughly depolymerized with the attainment of insoluble cellulose and water-soluble oxidized phenols and hemicelluloses. Hemicellulose is then separated from lignin by lowering the pH to 5.5 and by diluting the solution with ethanol, thus making the hemicellulose to flocculate. After the ethanol

removal the lignin components are recovered by decreasing the solution pH to 1.5-2.0 (Sun et al., 2000). The resulting lignin derivatives show, both, lower molecular weight and larger amount of carbonyl and carboxyl functionalities as compared to lignin extracted with common procedures, such as the Kraft or the sulfite methods (Holladay et al., 2007).

The aim of this work was to test the performance of lignin extracted with H_2O_2 at basic pH from miscanthus and giant reed, to act plant growth promoters on the germination and early growth of maize seedlings, thereby evaluating the effective biological activity of lignin components.

Materials & Methods

Biomasses. Miscanthus sample was provided by Phytatec (UK) Ltd and was harvested on February 2007 in Aberystwyth, Wales, UK. Giant reed cellulosic material came from the experimental farm located in Bellizzi (Province of Salerno, Italy) owned by the University of Naples "Federico II" and it was cropped on January 2010 (Pirozzi et al., 2010).

Chemical and reagents. Hydrogen peroxide (H_2O_2) was purchased from Merck KgaA. Ethanol was obtained from Carlo Erba Reagenti. Sodium hydroxide (pellet) was purchased from Clen Consult International SpA and it was reagent grade.

Alkaline oxidative hydrolysis. The alkaline oxidative extractive method has already been described elsewhere (Sun et al., 2000). Briefly, 5.0 g of samples were placed in a 150 mL of distilled water containing a 2 % H_2O_2 (v/v) solution. Then, the pH of the mixture was raised to 11.5 with a 4 M NaOH solution. The reaction was ended after 24 hours, by centrifuging the suspension (10,000 rpm x 10 min). The pH of supernatant (formed by lignin and hemicellulose) was lowered to 5.5 and 3 volumes of ethanol were added to the solution to make the hemicellulose to flocculate. The mixture was separated by filtrating it on cellulose filters. The ethanol present in the filtrate was, then, evaporated and the pH was lowered again to 1.5-2.0 to precipitate the lignin. Then, the

oxidized lignin was freeze-dried. The oxidized lignin were analysed by DFRC and, then, by ^{31}P-NMR spectroscopy. From now on, lignins extracted from miscanthus and giant reed will be referred to as MG and AD, respectively.

Elemental analysis. The elemental composition (C, N, H) of the lignin obtained from oxidative hydrolysis was carried out using an elemental analyzer EA 1108 Elemental Analyzer (Fisons' Instruments).

Bioactivity assay. The biological activity of lignin was evaluated by treating maize (*Zea mays*, L.) seeds with aqueous solution containing 0 (control), 0.1, 1, 10 and 100 ppm of C. After 96 h, germination percentage was evaluated by considering as germinated all the seeds with the testa disrupted and a radicle longer than 1 mm (Morrison et al., 1998). Then, seedlings were scanned with an Epson Perfection V700 modified flatbed scanner and primary root, lateral root and shoot length were measured with WinRhizo version 2012b (Regent Instruments, Inc.).

Statistical analysis. Yates' chi-square procedure was used to compare the germination percentages (Preacher, 2001), while Analysis of variance with One-Way ANOVA and Tukey's range test were performed to compare means among the treatments, by using Statistix, version 9 (Analytical Software, Tallahassee, FL). All the statistical analyses were carried out at a 95% confidence interval.

Results and Discussion

Elemental analysis. The results of the elemental analysis are reported in Table 1. The content of both carbon and hydrogen is higher in MG than in AD. Conversely, the amount of oxygen is greater in AD than in MG. Our results are compared with those of Kraft lignin and in lignosulfonates. The amount of oxygen is much lower in the reported Kraft lignin than in both the lignins extracted with oxidative alkaline hydrolysis. The percentage of oxygen in MG and AD is

40.7 and 45.6, respectively, while the relative amount of oxygen in Kraft lignin is around 28%. Also lignosulfonate extraction yields a less oxidized material (33.6% of oxygen) and some sulphur (*ca.* 6%) may be incorporated in it.

This clearly proves that the alkaline extraction method is very suitable for the production of much oxidized, highly water-soluble lignin.

Bioactivity assay. For the bioactivity assays the maize seeds were added with nutrient solutions of lignin derivatives based on increasing concentrations of phenol-derived organic carbon (OC). Even though the germination of maize seeds is not influenced by either MG or AD lignins for any of the tested concentration (Figure 1), both the applied lignin solutions have shown a positive biological activity on maize seedlings.

Overall the application of MG-lignin causes an initial increase of analysed growing parameters, with raising OC concentrations, up to a maximum of 10 ppm (Table 2). At larger OC amounts, both roots and shoot elongation are slightly reduced although also the more concentrated lignin solution still has provided a significant improvement of overall plant stimulation respect to control treatment (Table 2). Lignin from AD also positively affects both primary and lateral root development, while do not show any significantly differences on the shoot length (Table 2).

The maximum stimulating effect on primary root (PR) for MG-lignin has been recorded at OC concentration of 10 ppm, whereas only lower differences have been found among 0.1 and 1 ppm treatments and control solution (0 ppm). The 10 ppm treatment represents the optimum concentration also for lateral root (LR) growth and shoot elongation, which shows a sharp improvement equal to the 200% of increasing length for both the measured parameters.

For AD lignin, a significant enhancement of the PR length is revealed for the treatments at 1, 10 and 100 ppm, while the mean value obtained at 0.1 ppm do not differ from the control sample (Table 2). Similar results are found for the analysis of LR growth which shows an average increase

of 150% with 1, 10 and 100 ppm treatments compared to the control, whereas a slight stimulating effect has been recorded after the application of 0.1 ppm (Table 2).

According to our data, the germination of maize seeds is not influenced by the tested lignins, while they positively affected root and shoot growth of maize seedlings. These results appear in contradiction with previous reports, where an inhibitor effect of phenolic compounds on seed germination and plant growth have been described (Gerig & Blum, 1991; Chaves et al. 2000; Djurdjevic et al., 2004). However contrasting results have been reported on the interaction between phenolic components and earlier stages of physiological plant activity. Actually, as highlighted by Williams & Hoagland (1982), some phenolic compounds may just provide a variable time-delay effect instead of completely inhibit the seed germination. Moreover, the biological effect of phenolic molecules is strongly dependent.on both the chemical structures (Reigosa et al., 1999) and applied amounts. For instance Almaghrabi (2012), found a phytotoxic effect of four different phenolic substances (salyciclic, ferulic hydroxy benzoic and hydroxy phenyl acetic acid). According to this study, phenolic acids inhibited the germination of wild oat (*Avena fatua*, L.), but they did not affect the germination of wheat (*Triticum aestivum*, L.) and barley (*Hordeum vulgare*, L.). Also, Janovicek et al. (1997) showed that the toxic effect of phenolic acids on maize seed germination.was mainly related to the solute concentrations. Furthemore these authors have also reported that root length inhibition was not observed when coumaric and ferulic acids were tested conjointly on specific maize hybrids. Rasmussen & Einhellig (1977) reported an inhibitory effect of ferulic and *p*-coumaric acids, either alone or combined, until a maximum concentration. of 0.125 mM, over which both these phenolics behaved as weak stimulators of seedling growth.

The stimulation of biological activities on maize seedling revealed by the lignin extracts from MG and AD biomasses, is in line with recent findings about the positive influence of natural phenolic compounds on plant growth processes. In a comprehensive study on maize seed metabolism, Ertani and co-workers (2011b), tested two commercial biostimulants on maize seedling

development and on nitrogen and phenylpropanoid metabolic pathways. They found that both the products contained an appreciable amount of phenols and that the higher the rate of phenolic components the larger was the increase of root and the leaf dry weights., In a complementary experiment, the application of two commercial lignosulfonates raised the content of chlorophyll and the activity of the rubisco enzyme in maize treated plants, with an appreciable improvement of the photosynthesys rate,and a consequent increase of roots and leaves growth (Ertani et al. 2011a). Furthermore, Popa et al. (2008) reported that lignosulfonate and lignin from flax alkaline delignification greatly enhance the amount of green and dry biomass accumulated in bean (*Phaseolus vulgaris*, L.) stems. These authors have also noted a stimulating activity on mitotic division in the radicular meristems of bean, when plants were treated with lignin. This parameter is is an important variable for the plant development, since the mitotic rate is closely correlated with the physilogical activity related to plant growth and agronomical productivity.

The stimulatory effect of both MG and AD lignins may be associated to their hormone-like behaviour. The chemical nature of phenolic compounds plays a key role in determining their hormone-like activity. Pure solutions of diphenols, have shown to boost the activity of indolacetic acid (IAA), by reducing its oxidation by peroxidase (Pressey, 1990). On the contrary, monophenols promote this oxidation process (Schwertner & Morgan, 1966; Gross et al., 1977; Volpert et al., 1995), thus lowering the concentration of IAA and weakening the auxin activity on plant growth. Also different combinaton of matural phenolic components have revealed a positive activity toward the protection of IAA against its oxidative degradation (Volpert & Elstner, 1993). Interestingly, some polyaromatic compounds derived from gallic acid may also stimulate the development of plants. Negi et al. (2005) carried out an experiment where either an auxin (1-naphthalene acetic acid) or these gallic acid-derived molecules were tested together with a cytokinin (6-benzylamino purine). They found that the aromatic compounds were able to stimulate the growth of *Mentha arvensis*, L. likewise to the auxin in combination with cytokinin. Furthermore, Nardi et al. (2003)

proved that gallic, protocatechuic, *p*-hydroxybenzoic, vanillic and syringic acids have a stimulatory effect on the growth of silver fir (*Abies alba*, Mill.) seedlings. In particular they found a specific IAA-mimic activity for gallic and protocatechuic acids, while *p*-hydroxybenzoic acid, vanillic acid and syringic acid weeere mainly assoicated to a gibberellin (GA) like hormone. Pizzeghello et al. (2006) found that phenylacetic acid may also have IAA-like activity on silver fir plants. Also, Ertani et al. (2011a, b) proved that lignin-based biostimulants had both IAA- and GA-like activities. It is noteworthy the biological activity of HS and HULIS towards plant development is not exclusively related to the presence of phytohormones, but also for a direct hormone-like effect of lignin aromatic components on plant physiology. In a dedicated experiments Mora et al. (2010; 2012) treated cucumber plants with a humic acid with no detectable phytoregulators and reported a marked positive effect of HS on the overall plant growth. These authors claim that the hormone-like activity of HS is due to their molecular composition.The 13C-Nuclear Magnetic Resonance (NMR) spectra of HS revealed a molecular composition characterized by a large amount of aromatic compounds. Other authors (Muscolo et al., 1993, 1996; Nardi et al., 1994) have reported the IAA-like activity of HS on plant development, which has been related to the combination of a high content of both carboxylic and phenolic groups associated to humic fractions with an overall molecular weight lower than 3,500 Da. Since the lignins derivatives obtained in the present work had a large amounts of phenolic and carboxylic funcionalities, as showed by the 31-phoshorous and 13-carbon NMR spectra, and a molecular weigth lower than 3,000 Da, as highlitghted by the size exclusion chromatograms (chapters 3 and 4), it is conceivable that this lignin components may act as plant groth promoters similarly to bilogical acive humic fraction, thus improving the early stage of maize seedlings growth.

Conclusions

Biorefinery wastes are usually discarded or burnt. Finding a utlization for these waste materials is an important task, because they contain a high amount of still usable and marketable molecules. We propose to use these materials, which essentially are composed of phenolic mixtures, as plant growth promoters. Our lignins have stimulating effect on the early stage of growth of maize seedlings. Both of them are able to promote the elongation of the primary and lateral roots. Instead, an elongating effect on shoots was also reported for the lignin extracted from miscanthus. All these effect have been proved to be concentration-dependent. As a matter of fact, we registered different effects as lignin concentration has changed, with the greatest effect at 10 ppm for both the lignins and for all the parameters considerd.

Based upon previous studies on similar materials which showed a IAA- and GA-like activity of phenolic-made substrates used, it can be stated that our materials possess hormone-like activity. Also, since phenolic mixtures can stimulate the production on phenolic compounds inside the plants, these substrates may induce a greater resistance to abiotic stresses, due to the antioxidant activity of the produced phenols. For these reasons, our lignins could function as plant growth promoters. Withal, since the utilization of these materials hinders the burning of precious photosynthetate and prevents a further increase of greenhouse gases in the atmosohere, the employment of these materials greatly enhances the susainability of the biorefinery production.

Table 1. Elemental content[a] (%) of C, N, H, O and S in soluble lignin separated from MG and AD, as compared to that of a technical Kraft lignin.

Lignin	C	H	N	O	S
MG	53.8	5.5	0.6	40.1	0
AD	50.9	3.1	0.4	45.6	0
Technical Kraft lignin[b]	65.0	5.4	0.5	28.2	1.25
Lignosulfonate[c]	54.6	5.9	0	36	5.9

a. results are expressed on ash free basis

b. from El Mansouri & Salvadò, 2006.

c. from Holladay et al., 2007

Table 2. Percentage (%) length of primary root, lateral root and shoot for maize seedlings treated with aqueous solutions (mg C L^{-1}) of soluble lignin separated from MG and AD. Data are normalized to values found for control solution set as 100. Different letters in the columns indicate significant differences at 0.05 probability level.

	Primary root		**Lateral root**		**Shoot**	
Treatments	**MG**	**AD**	**MG**	**AD**	**MG**	**AD**
Control	100 c	100 c	100 b	100 c	100 b	100 a
0.1	122 bc	102 c	115 b	119 bc	134 b	99 a
1	107 bc	119 ab	108 b	156 a	125 b	93 a
10	177 a	140 a	207 a	161 a	235 a	110 a
100	138 b	119 ab	135 b	142 ab	131 b	95 a

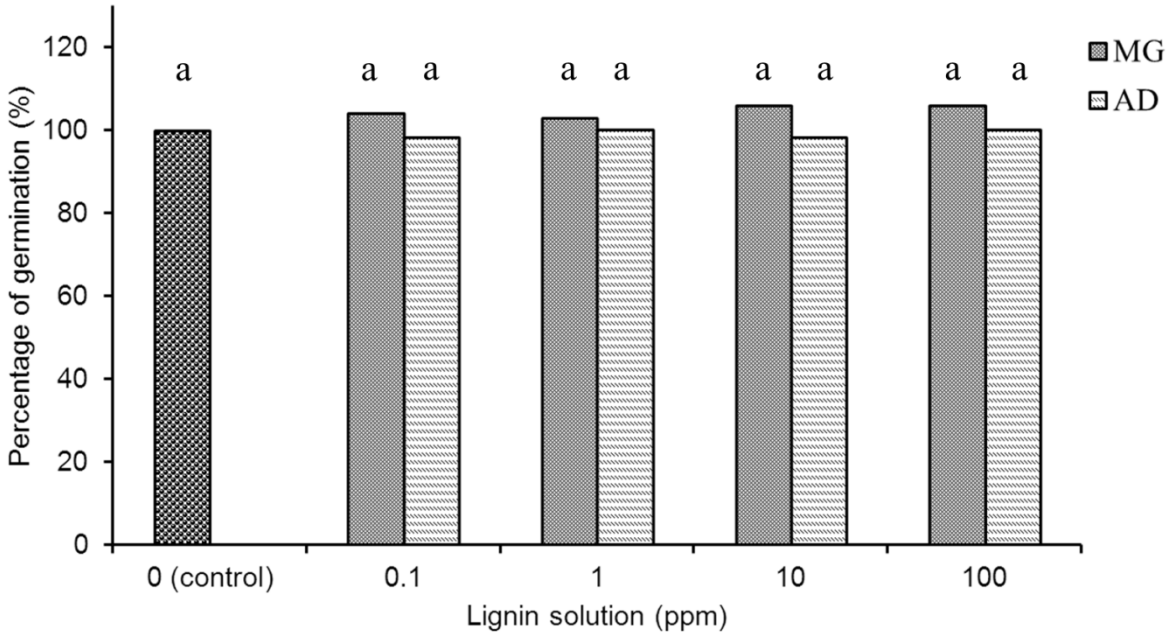


Figure 1. Percentage (%) of germination of maize seedlings as treated with aqueous solutions (mg C. L^{-1}) of soluble lignin separated from miscanthis (MG) and giant reed (AD) biomass. Data are normalized to values found for control solution set as 100.

References

Almaghrabi , O A (2012) Control of wild oat (*Avena fatua*) using some phenolic compounds I – Germination and some growth parameters. Saudi Journal of Biological Sciences, 19, 17-24.

Angelini, L G; Ceccarini, L; Bonsri, E (2005) Biomass yield and energy balance of giant reed (*Arundo donax*, L.) cropped in central Italy as related to different management practices European Journal of Agronomy 22, 375–389.

Atiyeh, R M; Lee, S; Edwards, C A; Arancon, N Q; Metzger, J D (2002) The influence of humic acids derived from earthworm-processed organic wastes on plant growth. Bioresource Technology, 84, 7-14.

Busato, J G; Zandonadi, D B; Dobbss, L B; Façanha, A R; Canellas, L P (2010) Humic substances isolated from residues of sugar cane industry as root growth promoter. Scientia Agricola, 67, 206-212.

Canellas, L P; Olivares, F L; Okorokova- Façanha, A L; Façanha, A R (2002) Humic Acids Isolated from Earthworm Compost Enhance Root Elongation, Lateral Root Emergence, and Plasma Membrane H+-ATPase Activity in Maize Roots. Plant Physiology, 13, 1951-1957.

Canellas, L P; Piccolo, A; Dobbss, L B; Spaccini, R; Olivares, F L; Zandonadi, D B; Façanha, A R (2010) Chemical composition and bioactivity properties of size-fractions separated from a vermicompost humic acid. Chemosphere, 78, 457-466.

Canellas, L P; Spaccini, R; Piccolo, A; Dobbss, L B; Okorokova- Façanha, A L; Santos, G A; Olivares, F L; Façanha, A R (2009) Relationships between chemical characteristics and root growth promotion of humic acids isolated from bazilian oxisols. Soil Science, 174, 611-620.

Canellas, L P; Zandonadi, D B; Busato, J G; Baldotto, M A; Simoes, M L; Martin-Neto, L; Façanha, A R; Spaccini, R; Piccolo, A (2008) Bioactiviy and Chemical Characteristics of Humic Acids from Tropical Soils Sequence. Soil Science, 179, 624-637.

Chaves, , N; Sosa, T; Alìas, J C; Escudero, J C (2001), Identification and Effect of Interaction Phytotoxic Compounds from exudates of *Cistus ladanifer* Leaves. Journal of Chemical Ecology, 27, 611-621.

Cherubini, F (2010) The biorefinery concept: Using biomass instead of oil for producing energy and chemicals. Energy Conversion and Management 51, 1412–1421.

Djurdjević, L; Dinić, A; Pavlović, P; Mitrović, M; Karadžić, B; Tešević; V (2004) Allelopathic potential of *Allium ursinum* L. Biochemical Systematics and Ecology 32, 533-544.

Dobbss, L B; Medici, L O; Peres, L E P; Pino-Nunes, L E; Rumjanek, V M; Façanha, A R; Canellas, L P (2007) Changes in root development of Arabidopsis promoted by organic matter from oxisols. Annals of Applied Biology, 151, 199-211.

El Mansouri, N-E & Salvadò, J (2006) Structural characterization of technical lignins for the production of adhesives: Application to lignosulfonate, kraft, soda-anthraquinone, organosolv and ethanol process lignins Industrial Crops and Products 24, 8–16.

Ertani, A; Francioso, O; Tugnoli, V; Righi, V; Nardi, S (2011a) Effect of Commercial Lignosulfonate-Humate on *Zea mays* L. Metabolism. Journal of Agricultural and Food Chemistry, 59, 11940 – 11948.

Ertani, A; Schiavon, M; Altissimo, A; Franceschi, C; Nardi, S (2011b) Phenol-containing organic substances stimulate phenylpropanoid metabolism in Zea mays. Journal of Plant Nutrition and Soil Science, 174, 496-503.

Eyheraguibel, B; Silvestre, J; Morard, P (2008) Effects of humic substances derived from organic waste enhancement on the growth and mineral nutrition of maize. Bioresource Technology, 99, 4206–4212.

Ferretti, M; Ghisi, R; Nardi, S; Passera, C (1991) Effect of humic substances on photosynthetic sulphate assimilation in maize seedlings. Canadian Journal of Soil Science, 71, 239–242.

García, A C; Santos, L A; Izquierdo, F G; Sperandio, M V; Castro, R N; Berbara, R L L (2012) Vermicompost humic acids as an ecological pathway to protect rice plant against oxidative stress. Ecological Engineering, 47, 203-208.

Gerig, T & Blum, U (1991) Effects of mixtures of four phenolic acids on leaf area expansion of cucumber. Journal of Chemical Ecology, 17, 29-40.

Gould, J M (1984). Alkaline peroxide delignification of agricultural residues to enhance enzymatic saccharification. Biotechnology and Bioengineering, 26, 46-52.

Gross, G G; Janse, C; Elstner, E F (1977) Involvement of malate, monophenols, and the superoxide radical in hydrogen peroxide formation by isolated cell walls from horseradish (*Armoraciala pathfolia* Gilib.). Planta, 136, 271-26.

Hawker, J S; Bungey, D M (1976) Isocitrate lyase in germinating seeds of *Prunus dulcis*. Phytochemistry, 15, 79-81

Heaton E, Voigt T, Long S P (2004) A quantitative review comparing the yields of two candidate C4 perennial biomass crops in relation to nitrogen, temperature and water. Biomass and Bioenergy, 27, 21–30.

Heaton, E A; Clifton-Brown, J; Voigt, T; Jones M B; Long S P (2004a) Miscanthus for renewable energy generation: European Union experience and projections for Illinois.

Holladay, J E; Bozell, J J; White, J F; Johnson, D (2007). Top Value-Added Chemicals from Biomass Volume II—Results of Screening for Potential Candidates from Biorefinery Lignin. Pacific Northwest National Laboratory, U. S. Dempartment of Energy.

Janovicek, K J; Vyn, T L; Voroney, R P; Allen, O B (1997) Early corn seedling growth response to phenolic acids. Canadian Journal of Plant Science, 77, 391-393.

Kesba, H H & El-Betagi, H S (2012) Biochemical changes in grape rootstocks resulted from humic acid treatments in relation to nematode infection. Asian Pacific Journal of Tropical Biomedicie, 2, 287-293.

Khanuja, S P S; Darokar, M P; Mishra, S; Gangwar, A; Shasany, A K; Kumar, T R S; Saikia, D; Kumar, S (2001) Development of a novel plant system as biosensor for detecting environmental hazards and bioactive molecules through distinct responses. Journal of Environmental Pathology, Toxicology and Oncology, 20, 15-22.

Lewandowski, I; Clifton-Brown, J C; Scurlock, J M O; Huisman W (2000) Miscanthus: European experience with a novel energy crop. Biomass and Bioenergy, 19, 209–27.

Loffredo, E; Berloco, M; Senesi, N (2008) The role of humic fractions from soil and compost in controlling the growth in vitro of phytopathogenic and antagonistic soil-borne fungi. Ecotoxicology and Environmental Safety, 69, 350–357.

Lopez-Capel, E; Sohi, S P; Gaunt, J L; Manning, D A C (2005) Use of thermogravimetry-differential scanning calorimetry to characterize modelable soil organic matter fractions. Soil Science Society of America Journal, 69, 136–140.

Mansur, H S; Mansur, A A P; Bicallho, S M C M (2005) Key Engineering Materials, 284-286, 745-748.

Merlo, L; Ghisi, R; Rascio, N; Passera, C (1991) Effects of humic substances on carbohydrate metabolism of maize leaves. Canadian Journal of Plant Science, 71, 419–425.

Mora, V; Bacaicoa, E; Zamarreño, A M; Aguirre, E; Garnica, M; Fuentes, M; García-Mina, J M (2010) Action of humic acid on promotion of cucumber shoot growth involves nitrate-related changes associated with the root-to-shoot distribution of cytokinins, polyamines and mineral nutrients. Journal of Plant Physiology, 167, 633–642.

Mora, V; Baigorri, R.; Bacaicoa, E; Zamarreno, A M; Garcia-Mina, J M (2012) The humic acid-induced changes in the root concentration of nitric oxide, IAA and ethylene do not explain the changes in root architecture caused by humic acid in cucumber. Environmental and Experimental Botany, 76, 24-32.

Morrison, D A; McClay, C; Porter, C; Rish, S (1998) The Role of the Lens in Controlling Heat-induced Breakdown of Testa-imposed Dormancy in Native Australian Legumes. Annals of Botany 82, 35–40.

Muscolo, A; Concheri, G; Nardi, S (1993) Effect of earthworm humic substances on esterase and peroxidase activity during growth of leaf explants of *Nicotiana Plumbaginifolia*. Biology and Fertility of Soils, 15, 127 – 131.

Muscolo, A; Felici, M; Concheri, G; Nardi, S (1993) Effect of earthworm humic substances on esterase and peroxidase activity during growth of leaf explants of *Nicotiana plumbaginifolia*. Biology and Fertility of Soils, 15, 127–131.

Muscolo, A; Panuccio, M R; Abenavoli, M R; Concheri, Nardi, S (1996) Effect of molecular complexity and acidity of earthworm faeces humic fractions on glutamate dehydrogenase, glutaminesynthetase, and phosphoenolpyruvate carboxylase in *Daucus carota α* II cells. Biology and Fertility of Soils, 22, 83 –88.

Nardi, S; Muscolo, A; Vaccaro, S; Baiano, S; Spaccini, R; Piccolo, A (2007) Relationship between molecular characteristics of soil humic fractions and glycolytic pathway and krebs cycle in maize seedlings. Soil Biology and Biochemistry, 9, 3138-146.

Nardi, S; Panuccio, M R; Abenavoli, M R; Muscolo, A (1994) Auxin-like effect of humic substances extracted from faeces of *Allolobophora caliginosa* and *Allolobophora rosea*. Soil Biology and Biochemistry, 26, 1341 – 1346.

Nardi, S; Pizzeghello, D; Bragazza, L; Gedol, R (2003) Low-Molecular-Weight Organic Acids and Hormone-Like Activity of Dissolved Organic Matter in Two Forest Soils in N Italy. Journal of Chemical Ecology, 29, 1549-1564.

Nardi, S; Pizzeghello, D; Gessa, C; Ferrarese, L; Trainotti, L; Casadoro, G (2000) A low molecular weight humic fraction on nitrate uptake and protein synthesis in maize seedlings. Soil Biology & Biochemistry, 32, 415–419.

Negi, A S; Darokar, M P; Chattopadhyay, S K; Garg, A; Bhattacharya, A K; Srivastava, V; Khanuja, S P S (2005) Synthesis of a novel plant growth promoter from gallic acid. Bioorganic & Medicinal Chemistry Letters, 15, 1243–1247.

Nelson, D L; Cox, M M. Lehninger Principles of Biochemistry. W. H. Freeman & Company, 6th edition, 2012.

Oku, T; Sadako, N (2002) Digestion, absorption, fermentation, and metabolism of functional sugar substitutes and their available energy. Pure and Applied Chemistry, 74, 1253–1261.

Piccolo, A (2002) The supramolecular structure of humic substances: a novel understanding of humus chemistry and implications in soil science. Advances in. Agronomy, 75, 57–154.

Pirozzi D, Yousuf A, Fagnano M, Mori M, Fiorentino N, Impagliazzo A (2010) Lipids production by yeast grown on giant reed biomass. Journal of Biotechnology , 150, S1, 167-168.

Pizzeghello, D; Zanella, A; Carletti, P; Nardi, S (2006) Chemical and biological characterization of dissolved organic matter from silver fir and beech forest soils. Chemosphere, 65, 190–200.

Popa, V I; Dumitru, M; Volfa, I; Anghel, N (2008) Lignin and polyphenols as allelochemicals. Industrial crops and products, 27, 144–149.

Preacher, K. J. (2001, April). Calculation for the chi-square test: An interactive calculation tool for chi-square tests of goodness of fit and independence [Computer software]. Available from http://quantpsy.org.

Pressey, R (1990) Anions activate the oxidation of IAA by peroxidase from tomato and other sources. Plant Physiology, 93, 798-804.

Puglisi, E; Fragoulis, G; Ricciuti, P; Cappa, F; Spaccini, R; Piccolo, A; Trevisan, M; Crecchio, C (2009) Effects of a humic acid and its size-fractions on the bacterial community of soil rhizosphere under maize (*Zea mays* L.). Chemosphere, 77, 829-837.

Rasmussen, J A & Einhellig, F A (1977) Synergistic Inhibitory Effect of p-Coumaric and Ferulic Acids on Germination and Growth of Grain Sorghum. Journal of Chemical Ecology, 3, 197-205.

Reigosa, M J; Souto, X C; Gonzàlez, L (1999) Effect of phenolic compounds on the germination of six weeds species. Plant Growth Regulation 28, 83–88.

Robertson, R A; Irvine, J C; Dobson, M E (1907) A Contribution to the Chemistry and Physiological Action of the Humic Acids. Biochemical Journal, 2, 458-482.

Schwertner, H A; Morgan P W (1966) Role of IAA-Oxidase in Abscission Control in Cotton. Plant Physiology, 41, 1513-1519.

Silva-Matos, R R S; Cavalcante, I H L; Jùnior, G B S; Albano, F G; Cunha, M S; Beckman-Cavalcante, M Z (2012) Foliar Spray of Humic Substances on Seedling Production of Watermelon cv. Crimson Sweet. Journal of Agronomy, 11, 60-64.

Simpson, A J (2002) Determining the molecular weight, aggregation, structures and interactions of natural organic matter using diffusion ordered spectroscopy. Magnetic Resonance in Chemistry, 40, 572–582.

Sladky, Z (1959) The effect of extracted humus substances on growth of tomato plants. Biologia Plantarum, 1, 142–150.

Smiricky-Tjardes, M R; Flickinger, E A; Grieshop, C M; Bauer, L L; Murphy, M R; Fahey Jr, G C (2003) In vitro fermentation characteristics of selected oligosaccharides by swine fecal microflora. Journal of Animal Science, 81, 2505–14.

Spaccini, R; Zena, A; Piccolo A (1998) Molecular differences in Soil Organic Matter fractions obtained by selective extractants as revealed by DRIFT infrared spectroscopy. Fresenius Environmental Bulletin, 7, 458-465.

Stevenson, F J Humus Chemistry: Genesis, Composition, Reactions, 2nd ed.; John Wiley & Sons, Ltd: New York, 1994.

Sun, R, Tomkinson, J, Wang, S; Zhu, W (2000). Characterization of lignins from wheat straw by alkaline peroxide treatment. Polymer Degradation and Stability 67, 101-109.

Sun, Y & Cheng, J (2002) Hydrolysis of lignocellulosic materials for ethanol production: a review. Bioresource Technology, 83, 1–11.

Tufencki, S; Turkmen, O; Sonmez, F; Erdinc, C; Sensoy, S (2006) Effects of humic acid doses and application times on the plant growth, nutrient and heavy metal contents of lettuce grown on sewage sludge-applied soils. Fresenius Environmental Bulletin 15, 295–300.

Vaughan, D & MacDonald, I R (1976) Some effects of humic acid on cation uptake by parenchyma tissue. Soil Biology & Biochemistry, 8, 415–421.

Vaughan, D & Malcom, R E (1985) Influence of humic substances on biochemical processes in plants. In: Vaughan, D & Malcom, R E (Eds.), Soil Organic Matter and Biological Activity, Martinus Nijhoff/Junk W, Dordrecht, The Netherlands, pp. 77–108.

Vaughan, D & Malcom, R E (1985) Influence of humic substances on growth and physiological processes. In: Vaughan, D & Malcom, R E (Eds.), Soil Organic Matter and Biological Activity, Martinus Nijhoff/Junk W, Dordrecht, The Netherlands, pp. 37–76.

Verlinden, G; Pycke, B; Mertens, J; Debersaques, F; Verheyen, K; Baert, G; Bries, J; Haesaert, G (2009) Application of Humic Substances Results in Consistent Increases in Crop Yield and Nutrient Uptake. Journal of Plant Nutrition, 32, 1407-1426.

Volpert, R & Elstner, E F (1993) Biochemical activities of propolis extracts. I. Standardization and antioxidative properties of ethanolic and aqueous derivatives. Zeitschrift für Naturforschung C 48, 851-857.

Williams, R D & Hoagland, R E (1982) The Effects of Naturally Occurring Phenolic Compounds on Seed Germination. Weed Science, 30, 206-212.

Zakzeski, J; Bruijnincx, P C A; Jongerius, A L; Weckhuysen, B M (2010) The Catalytic Valorization of Lignin for the Production of Renewable Chemicals. Chemical Reviews, 110, 3552–3599.

Chapter 6

Conclusions

In this research, lignin from three biomasses (miscanthus, giant reed and a lignocellulosic residue from giant reed) was extracted by two different methods and extensively characterised. The extraction methods involved the use of either sulfuric acid (SAL) or hydrogen peroxide at alkaline pH (Ox). After isolation and characterization, the biological activity of lignin fragments was assayed on maize seed germination and maize seedling growth.

Gravimetrical data showed that the Ox treatment produced low extraction yields. This was attributed to either accelerated mineralization of fragmented lignin material or still poor optimization of the extraction process. Conversely, the SAL method provided an even greater lignin yield than that determined by the Klason method, possibly due to condensation between carbohydrates and lignin structure during the acidic reaction.

Thermal analysis and IR and NMR spectra revealed the high degree of purity of extracted lignin and efficient removal of plant cell wall sugars, with exception of Ox-lignins, as revealed by NMR spectra and thermograms, where some residual carbohydrates were still present. Both the IR and the NMR spectra also showed a larger extent of oxidation in Ox-lignin materials in respect to the SAL-lignin samples. Furthermore, solid-state NMR measurements allowed us to evaluate relaxation time that revealed a greater molecular mobility of Ox-lignins than the more rigid SAL-lignin materials. In line with these findings, Ox-lignin samples resulted 15 times more soluble than SAL-lignin materials, thereby indicating that the lignocellulosic biomasses treated with H_2O_2 at alkaline pH produced more oxidized, fragmented and motile lignin residues.

High pressure size exclusion chromatograms (HPSEC) of Ox-lignin samples revealed, in fact, a generalized bimodal elution profile in which the molecules eluted at the low retention volume had a size lower than 3000 Da.

The molecular composition of Ox-lignins was determined by applying the DFRC fragmentation technique supported by GC-MS and a derivatization of lignin extract by 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxa-phospholane. The DFRC protocol selectively broke down ether linkages in lignin and produced hydroxylated molecules to be then acetylated and determined by GC-MS. Among monolignols produced by DFRC lignin fragmentation, the guaiacyl units were more abundant than other ones, especially for the miscanthus. The DFCR technique also indicated the presence of carbohydrates, though in very low amount, thus showing that the lignin extracts did not have complete purity (purity > 95% of the total chromatographic area). Finally, ^{31}P NMR spectroscopy revealed the occurrence of some phenolic acids, thus indicating that not all lignin fragments isolated by the employed method could be determined by GC. In fact, the DFRC-GC/MS method may not appreciate lignin fragments holding acidic functionalities produced during the alkaline oxidative process.

The lignin fragments obtained from the alkaline hydrolysis of miscanthus and raw giant reed where used as humic-like materials to verify their stimulating effect on the germination of maize seeds and on the early stages of maize seedlings. Our results showed that extracted lignins had a significantly positive effect on elongation of both primary and lateral roots. This has been proved to be a concentration-dependent effect, with the maximum of elongation measured at a humic-like concentration of 10 ppm, for both lignin biomasses. Moreover, lignin from miscanthus enhanced shoot elongation, whereas lignin from giant reed had no effect on this parameter. Altogether, these results indicate that lignin exerted a hormone-like effect on plant development. This may signify

that application of lignin fragemnts to plants may encourage the production on phenolic compounds inside plants, thus improving their resistance to both biotic and abiotic stresses.

This research showed that water-soluble lignin residue could be separated from lignocellulosic biomasses employed in biorefinery processes and may become an added-value agrochemical to increase plant production. The sulfuric acid treatment was efficient in completely hydrolyze carbohydrates and leave a pure lignin, but it hardly produced a depolymerized and water-soluble lignin. Conversely, the oxidative alkaline method, though low in yield, successfully produced a depolymerized and carboxyl-rich lignin that resulted totally soluble in water. The water-soluble lignins isolated from miscanthus and raw giant reed were proved to act as humic-like materials and be effective in the early-stage stimulation of plant growth, thus promising their massive use in agriculture as natural agrochemicals. Further studies on the activity of these materials on the overall develompent of plant are anyhow needed in order to prove their plant growth stimulation also in more advanced plant growth stages.